Isolation and Characterization of Novel Mycobacteria Species from *Sarracenia purpurea*

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Minh Phuong Thi Tran

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John L. Dahl, Ph.D.

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List of Abbreviations

LEC	Laurentian Environmental Center
SPSP	Savanna Portage State Park
NJ	Neighbor Joining
ML	Maximum Likelihood
MLSA	Multilocus Sequence Analysis
OD_{600}	Optical density at a wavelength of 600 nm
DPA	Dipicolinic Acid
OD_{440}	Optical density at a wavelength of 440 nm

Chapter 1: Introduction

Mycobacteria are an ancient bacterial genus intricately linked with human history, the environment, and disease prevalence. The genus belongs to the phylum Actinobacteria, order Actinomycetales and is most closely related to the *Corynebacterium* and *Nocardia* genera [1,2]. Mycobacteria were first officially described by Armauer Hansen while treating leprosy patients in 1874 and by Robert Koch's 1882 work with tuberculosis patients [3]. Thereafter, mycobacteria discoveries started emerging in a range of hosts such as fish, ruminants, and fowl along with different environments like sphagnum bogs, lakes, and soil. By 1896 the genus *Mycobacterium* was adopted by the scientific community [1,4].

Of the 165 accepted mycobacteria species, the majority are environmental mycobacteria while a small minority are host-associated organisms. Environmental mycobacteria, sometimes referred to as nontuberculous mycobacteria, are those that do not have a host reservoir [1,5]. Mycobacteria are small, acid-fast, pleomorphic bacteria uniquely characterized by a thick outer cell wall composed of mycolic acids. The lipid rich cell wall provides innate resistance to most antibiotics, disinfectants, and environmental assaults [6,7]. Most environmental species are free-living saprophytes meaning nutrients are obtained from dead organic matter [8,9]. In contrast, pathogenic species are obligate parasites acquiring host macromolecules for nutrients [10]. The genus can also be broken into two general categories based on growth rate: slow-growers (≥7 days to visualize colonies on culture media) and fast-growers (3-6 days to visualize colonies). The majority of environmental species are fast growers, whereas pathogenic species tend to be slow growers [1].

Research has primarily focused on the few highly pathogenic species until the mid to late 20th century when it became clear several environmental species were also opportunistic pathogens [11]. These opportunistic species caused disease in sick or immunocompromised persons such as cervical lymphadenitis in children and secondary pulmonary infections in people with AIDS [12,13]. It is now understood that of the 32 known pathogenic and

opportunistic pathogenic species, at least 16 have been found in the environment [1,3,14]. Other reports state up to 24 different environmental mycobacteria species have been implicated in causing opportunistic infections [5,8]. This indicates that the majority of nontuberculous mycobacteria-related infections have latent reservoirs in the environment. It has also been shown that variable efficacy (0-80% effectiveness) in the *Mycobacterium bovis* Calmette-Guerin (BCG) vaccine against pulmonary tuberculosis is primarily attributed to previous exposure to environmental mycobacteria [15–17]. When exposed to environmental mycobacteria, the human immune system can stimulate a small but productive cell-mediated response [16]. When challenged with the BCG vaccine, the already present immune response either masks the effects of the BCG vaccine or prevents the BCG vaccine from creating an even more productive and appropriate immune response. This results in an immune response inadequately effective against later *M. tuberculosis* infections [16]. Observational evidence for this phenomena is the finding that the effectiveness of the BCG vaccine varies greatly by geography (high environmental mycobacteria vs low environmental mycobacteria regions) and age of vaccination. The BCG vaccine is highly ineffective in regions with high environmental mycobacteria populations, and provides productive immunity in children but not in adults. Unfortunately, health risks due to the association between environmental mycobacteria, opportunistic infections, and interference with the BCG vaccine have not garnered much attention. The great overlap of environmental and pathogenic species and this implication for public health warrants further examination of mycobacteria in their various environments.

1.1 The Environments

Mycobacteria have been found almost ubiquitously in soil and water environments. Soil environments range from high nitrogen potting soils to acidic swamps and bogs [1,5]. Notably, several mycobacteria species have also been found in landfill soils [18]. Aquatic environments containing these bacteria include natural systems such as lakes, streams,

rivers, and manmade systems like chlorinating water treatment facilities and institutional water systems [5,14]. Moist instruments such as showerheads, catheters, and bronchoscopes have also been shown to contain mycobacteria [19,20]. Mycobacterial-inhabited environments share many abiotic and biotic factors such as low oxygen, low to neutral pH, and low nutrient concentrations along with areas lacking faster growing microbes (Kazda, 2000, *personal observation*). These environments are often uninhabited by other bacteria because of inadequate nutrient and oxygen concentrations for replication, but mycobacteria have evolved to thrive in such environments [5].

Mycobacteria have evolved complex mechanisms for survival in its various habitats. Genomic analysis suggests the majority of pathogenic mycobacteria species independently evolved from environmental species, which may be why many survival mechanisms are shared between pathogenic and environmental mycobacteria [21,22]. Therefore, the evolution of successful virulence traits in mycobacteria likely first occurred in response to environmental stress, competition for resources, and predation [1,22,23].

It has been proposed that one specific environment, sphagnum peat bogs, could act like a 'pathogen training ground' for mycobacteria [22]. Indeed, De Groote *et al*. (2006) reported mycobacteria opportunistic infections that were caused by exposure to commercial peat potting soil [24]. Observations that waterborne mycobacteria and phagocytic amoebae often inhabit the same environmental niches led to *in vitro* studies that revealed some environmental mycobacteria were resistant to phagocytosis and could survive within the phagosome of macrophages [25,26]. Amoebae are analogous to human macrophages because of the shared ability to perform phagocytosis and kill bacteria [27]. It has been well documented that pathogenic mycobacteria also evade the phagocytic process whereby it then survives within the phagosomes [28–32]. Regardless of the similarities between amoeba and macrophage predation on mycobacteria, few studies have focused on mycobacterial ecology. However, mycobacteria that are natural inhabitants of bogs and pitcher plants can be excellent model organisms to study pathogenic mycobacteria because of the similarities between the microenvironments of bogs and human macrophages. Because of

the close evolutionary relationship between pathogenic and environmental mycobacterial species, characterizing environmental species could provide insights into the mycobacterial survival mechanisms in humans.

1.2 The Bogs and the Pitchers

Sphagnum peat bogs are found on every inhabited continent [1,33]. In northern latitudes, bogs are characterized by a thick peat layer covered by sphagnum moss (*Sphagnum* spp.), sedges, and little other vegetation. The environment becomes waterlogged due to high peat levels, quickly creating an anoxic environment below the moss. The moist, oxygen-deprived environment decreases decomposition rates making bogs an abundant source of carbon [34]. Cation and nutrient levels, particularly calcium, phosphate, and nitrogen, are extremely low due to lack of nutrient-rich run off into the bogs along with constant nutrient uptake from local vegetation [33,35,36].

Sphagnum moss and peat soil are the main ion and nutrient regulators within the bogs. Sphagnum moss intakes various cations and releases H^+ ions into the water while peat decomposition releases organic acids, such as humic acid [1]. Thus, cations are a limiting resource to other organisms and surface water pH is maintained at 4.2 or lower with soil pH resting between 3-5 [35,36]. The high proton concentration creates an acidic environment that is disruptive and detrimental to most micro-organisms' metabolic processes, but is somehow tolerated by mycobacteria. Along with organic acids, peat decomposition also releases the amino acids glutamate and aspartate, which are also necessary for mycobacterial growth [1]. Mycobacteria are typically found in the intermediate layers between the sphagnum head and peat layer where they are protected from solar UV radiation but can access oxygen. Mycobacteria have been found in numerous European and American bogs, but their role within the bog ecology and with the bog vegetation has rarely been studied [1,37]. A new species, *Mycobacterium minnesotense*, was recently discovered in northern Minnesota sphagnum peat bogs and is indicative of novel mycobacterial species awaiting discovery [38].

While sphagnum moss and sedges are the most abundant vegetation in northern Minnesota peat bogs, the most commonly occurring forb is the carnivorous pitcher plant (*Sarracenia purpurea*) (30-60% frequency), with conifers and low-lying shrubs in lower frequencies [36]. *S. purpurea* produce hydrolytic enzymes to digest prey, but do not produce chitinase required to breakdown chitin in arthropod exoskeletons [39–41]. Therefore it must partially rely on a self-contained microecosystem harboring a fully-composed aquatic food web to degrade its arthropod prey [39]. *S. purpurea* food web communities are diverse and vary geographically, but most commonly found eukaryotes are dipterans, mites, rotifers, and a keystone predator the *Wyeomyia smithii* larvae [39]. The bacterial community has been less studied, but the presence of *Bacillus*, *Lactococcus*, *Rhodospirillum*, and members of the *Enterobacteriaceae* family have been observed in pitchers through molecular sequencing [39,42]. Currently, there are no known studies that have identified mycobacteria as part of the pitcher plant community. It can be conceived that if mycobacteria are in the bogs they may also be residing in the pitcher plants. Pitcher plant waters are of interest because it is a mostly isolated system and the food web is relatively small allowing for controlled *in vivo* studies of bacteria. If mycobacteria reside in the pitcher plants then it would be a valuable tool for studying mycobacteria ecology.

Prior to this thesis work, mycobacteria have not been identified in *S. purpurea* pitchers. Therefore, it is the goal of this research to determine if pitcher plants are a unique environmental niche harboring mycobacteria, and if so, to determine if the mycobacteria exhibit unique stress responses. To answer this question, a polyphasic approach was utilized combining phenotypic (biochemical properties, fatty acid analysis) and genotypic (gene sequencing, phylogenetic analysis) testing methods.

Chapter 2: Isolation and Identification of Mycobacteria

2.1 Introduction

Mycobacteria inhabit a diverse set of environments reflecting the diversity found within the genus. The single most common biotope harboring mycobacteria are sphagnum peat bogs, where more than 26 different species from four continents have been isolated [1]. Although sphagnum peat bogs are common mycobacterial ecosystems, species exhibit location specificity and limited dispersal. For instance, *M. cookii* can only be found on New Zealand, *M. madagascariense* is found on Madagascar, and *M. komossense* resides in boreal climates of Scandinavia and North America [1,37]. To date, it is not clear why there is such geographic variation in the mycobacteria species, but it is suspected that environmental differences do not play a significant role [1]. Despite the geographic differences, all bogs containing mycobacteria share common characteristics such as types of vegetation, isolation from groundwater, and water pH.

There are many variations in bog types, but the only bogs where mycobacteria have been found are sphagnum peat bogs [1,36]. These bogs always exhibit low plant diversity with no access to standing groundwater and the presence of sphagnum and sedge species. The presence of specific species of sphagnum help determine mycobacteria diversity. Mycobacteria densities are higher when bogs are comprised of *Sphagnum balticum, Sphagnum recurvum,* or *Sphagnum tenellum* and lower in *Sphagnum imbricatum* or *Sphagnum magellanicum* bogs [1]. Minnesota sphagnum peatland–classified as either northern open bogs or northern poor fens which become spruce bogs–contain *S. magellanicum* and *Sphagnum angustifolium* [36]. Therefore, it is expected mycobacteria diversity will be lower in Minnesota bogs when compared to other bogs [37,43]. Minnesota peatlands are characterized by a limited number of forbs, grasses, and shrubs with indicator species being pitcher plants (*S. purpurea*), fen wiregrass sedge (*Carex lasiocarpa*), and leatherleaf (*Chamaedaphne calyculata*) in poor fens. Open bogs are dominated by pitcher plants (*S. purpurea*), leatherleaf (*C. calyculata*), bog laurel (*Kalmia*

polifolia), and Labrador tea (*Ledum groenlandicum*) [36]. Since pitcher plants are found in both poor fens and open bogs, they were appropriate for further study.

S. purpurea are naturally found throughout North America. They range from southeastern United States up the Atlantic coast, then west along the United States–Canada border to northern British Columbia [44]. Variation in biotic factors influencing its growth range results in differences in habitats, growth patterns, and food web assemblages. For instance, *S. purpurea* growing in the southern region grow year round in pine savannahs while northern growing *S. purpurea* have a shorter growth period in sphagnum peat bogs. Food web assemblages are relatively constant throughout the region, but species richness increases with increasing latitude [44]. Therefore, Minnesota pitcher plants likely harbor a rich aquatic invertebrate-protozoan-bacterial community. Currently, no studies have examined the food web assemblages of Minnesota pitcher plants.

S. purpurea aquatic communities have been shown to differ between different geographic sites, and even between pitchers in the same location [39,44]. Therefore, high variability exists in the bacterial assemblages of different pitchers. Furthermore, microhabitats in the pitchers–the upper water layer, the lower sediment layer, and the pitcher walls–are also known to harbor different species of bacteria [42]. Since environmental mycobacteria are known saprophytes, the water layers were sampled and the pitcher walls were not. In this first chapter, I present the experiments examining the hypothesis that mycobacteria are inhabitants of northern Minnesota pitcher plants based on the previous findings of mycobacteria in Minnesota sphagnum bogs. Results indicate that mycobacteria are found in Minnesota *S. purpurea* pitchers and are 1) not found to be the same mycobacteria in the immediate surrounding area and 2) likely two novel species based on phylogenetic relationships.

2.2 Materials and Methods

2.2.1 Characteristics of the Study Sites and Samples

Samples were collected from 43 pitchers from four Minnesota floating sphagnum bogs: Repose Lake north of Ely, MN, the Laurentian Environmental Center (LEC) (47.57 N, 92.57 W), a local bog 15 miles north of Duluth (46.77 N, 92.12 W), and Savanna Portage State Park (SPSP) (46.82 N, 93.15 W) (Fig. 1). 3-6 mL aliquots of pitcher water was extracted on site and transferred to 15 mL conical tubes using transfer pipettes. pH of the water samples was recorded off site. Samples were maintained at 4°C until used.

All Middlebrook 7H9 media in this chapter was supplemented with 10% albumin dextrose catalase (ADC) (v/v), 0.05% Tween 80 (v/v) and 0.2% glycerol (v/v). All Middlebrook 7H11 media was supplemented with 10% oleic albumin dextrose catalase (OADC) (v/v) and 0.2% glycerol. These will be referred to as 7H9 and 7H11, respectively.

2.2.2 Mycobacteria Isolation

Mycobacteria isolation was conducted by modifying and combining previous procedures in order to remove spore-formers, fungi, fast-growing bacteria, and pH-sensitive bacteria [12,18]. Five-mL aliquots of nutrient broth was added directly to the water samples then incubated at 32°C for 2-4 hours with shaking. The solution was then centrifuged at 3,700 *g* for 15 min. The supernatant was removed and the pellet resuspended in 11-mL aliquots of mycobacteria selection buffer (3.6 mg/mL NaOH (w/v), 45 µg/mL cycloheximide, 0.115 µg/mL malachite green in dH_2O, pH 12) and incubated at room temperature for 20 min. The samples were centrifuged at 3,700 *g* for 15 min then the supernatant was removed. The pellet was resuspended in 11 mL acid buffer (0.2 M HCl–0.2 MKCl, pH 2) and incubated at room temperature for 20 min. Ten-mL aliquots of phosphate buffer (pH 6.9) was directly added and the solutions were centrifuged at 3,700 *g* for 15 min. Most of the resulting supernatant was removed and the pellet resuspended in the remaining solution. A 100-µL volume of each sample was plated on 7H11 additionally

supplemented with 17.5 µg/mL cycloheximide, and 250 µg/mL nalidixic acid per liter. Cycloheximide is used to inhibit protein synthesis in fungi and nalidixic acid will inhibit growth of Gram-positive and Gram-negative bacteria by inhibiting DNA synthesis, but not mycobacteria. All plates were incubated at 28°C and examined for growth at 3, 5, and 7 days and then every 2 weeks thereafter.

Ziehl-Neelsen acid-fast staining was conducted to detect the presence of mycobacteria from single colonies. All acid-fast stain-positive bacteria were assumed to be mycobacteria and designated a strain name starting with DL725. Through this method, 21 possible mycobacteria were isolated from pitcher water samples.

2.2.3 Gene Sequencing and Analysis

Gene sequencing and analysis was used to verify the identity of the isolated strains. Fresh cultures grown on 7H11 plates were swabbed and suspended in microcentrifuge tubes with tethered caps with 400 µL of 0.1-mm glass beads and filled with dH_2O. The samples were 'bead beaten' in a Bio101/Savant FastPrep FP120 for 45 sec to lyse cells and expose bacterial DNA. Alternatively, cells were hand-vortexed for 45 sec to release DNA. PCR was performed on the lysates for the following gene sequences: *dnaJ* with primers J10F and J335R, *hsp65* with primers Tb11 and Tb12, *secA1* with primers Mtu.Forward 1 and Mtu.Reverse 1, and *rpoB* with primers MycoF and MycoR (Table 1) [45–48]. PCR reaction mixtures contained 2-µL aliquots of the lysed solution, 1 µL of designated forward and reverse primers, 1 µL DMSO and 20 µL dH_2O in PCR tubes with illustra™ PuReTaq™ Ready-To-Go™ beads. Samples were considered nonmycobacteria if no amplified product was observed as a band around the predicted size after agarose gel electrophoresis. "Positive" samples were prepared for sequencing using a QIAprep PCR Purification Kit following manufacturer instructions. In the case of *dnaJ*, two bands were routinely presented in the gel and the band at the predicted product length (350 bp) needed to be cut from the gel using a sterile blade then processed using a QIAprep Gel Extraction Kit, following manufacturer instructions. After extraction, gel electrophoresis was performed for extraction verification. DNA concentration was optimized and

sequenced off-site at the University of Minnesota Biomedical Genomics Center and Analysis Facility (St. Paul, MN). Sequence editing was done using Sequence Scanner v1.0 (Applied Biosystems) and identified by nucleotide BLAST searches for homologous sequences (http://www.blast.ncbi.nlm.nih.gov). A species differentiation similarity cut-off of 97% was used. Of the 21 isolates, 18 were confirmed as belonging to the *Mycobacterium* genus.

2.2.4 Phylogenetic Analysis

Phylogenetic trees were constructed using the maximum likelihood (ML) and neighbor-joining (NJ) methods using the Kimura 2-parameter distance correction model because it is practical for large datasets and allows for bootstrapping. Main large nodes were generally respected in both analyses, but robustness as measured by bootstrapping was lower in the ML trees. Therefore, NJ was chosen as the primary method of analysis. For both analyses, sequences for each gene were aligned and ends trimmed to maintain a constant fragment length thereby increasing the reliability of the trees. Following alignment of the individual sequences, all sequences for each strain were combined to form a concatenated sequence in order: *rpoB-dnaJ-hsp65. secA1* was not used in the concatenate because too few known species have published *secA1* sequences available. Mycobacterial strains were not used in concatenate analyses if any of the gene sequences were missing for an isolate or type strain. In analyses with *M. minnesotense*, concatamers of *hsp65-dnaJ* were used. For the concatenate trees, *Nocardia farcinica* IFM 10152 and *Corynebacterium diptheriae gravis* NCTC 13129 were independently used as outliers. Preliminary trees using *N. farcinica,* which is a standard outlier in mycobacteria phylogeny studies, as an outlier resulted in its position consistently placed within the *Mycobacterium* genus. Therefore, *C. diptheriae gravis* was chosen as the outlier as it was consistently branched outside the *Mycobacterium* genus. All phylogenetic analysis was performed using MEGA6 [49]. Please refer to Table A1 in the appendix for all strain types and accession numbers use in phylogenetic analyses.

2.3 Results

2.3.1 Mycobacteria isolated from two regions

Acid-fast positive bacteria were isolated from pitcher plants found at Repose Lake and at the LEC, but not from SPSP or from the Duluth area. Of the isolates obtained, 12 were from Repose Lake and 15 were from LEC and were designated DL725 through DL751. The pitcher waters exhibited a pH range of 2-4 and contained large amounts of organic debris, invertebrates, and arthropods. Water temperatures in the pitchers were typically two degrees warmer than in the surrounding bog water. Both the pitcher and bog waters were 2-4 degrees warmer than ambient air temperature.

2.3.2 Sequence analysis of amplified gene regions

Internal regions from the genes *rpoB* (723 bp), *hsp65* (441 bp), *dnaJ* (350 bp), and *secA1* (700 bp) were chosen for identification of mycobacteria in pitcher waters. BLAST searches for homologous sequences were conducted and 18 isolates were positively verified as mycobacteria (Table 2). DL743-DL745 failed to amplify any genes and thus are likely not to be mycobacteria. Based upon *dnaJ* homology, DL748 was identified as *Rhodococcus erythropolis.* Homology for the RNA polymerase β-subunit indicated DL741, DL746, and DL749 were *Gordonia polyisoprenivorans VH2.* Both *Rhodococcus* and *Gordonia* are close relatives to *Mycobacteria* and weakly acid-fast. The remaining 18 isolates (DL725-DL739, DL742, DL750 and DL751) are indicated as being closely related to fast growing mycobacteria *M. flavescens, M. rhodesiae, M. vaccae* and M. sp. ATCC 25793, with *M. mageritense* having the highest similarity (95-96%) (Table 2). Every isolate had at least one gene sequence similarity below 95% (Table 2). No isolates were indicated by a 99-100% sequence similarity for any of the amplicons. Furthermore, there was no consistency in identified homologues between the genes for any given isolate. Therefore, this would suggest that a novel species was identified. To better determine the relationship between the isolates and their homologues, phylogenetic trees were constructed.

2.3.3 Phylogenetic analysis of amplified sequences

All phylogenetic analyses using *N. farcinica* IFM 10152 as an outlying group consistently branched this species within the *Mycobacterium* genus (Fig. 2). *N. farcinica* had to therefore be artificially designated as the outlying branch after each tree construction. This was an alarming find as most mycobacteria phylogenetic studies use this species for their outliers because of its close evolutionary relationship to mycobacteria and partial acid-fast properties, even though it is not a true outgroup as indicated in figure 2. Instead, *Corynebacterium diptheriae gravis* NCTC 13129, a more distant evolutionary group with partial acid-fast staining capabilities, was used as the outlier. *C. diptheriae gravis* behaved as an outlier with every tree construction without the need to artificially construct an outlier (Fig. 3).

All phylogenetic trees indicate the pitcher plant isolates are most closely related to *M. chelonae*, *M. flavescens*, *M. vaccae*, and *M. cookii*, all of which have previously been found in sphagnum bogs globally or in Minnesota [1]. These species are not known pathogens or opportunistic pathogens of humans. Branches were short on all phylogenetic trees, indicative of little genetic heterogeneity. The pitcher plant isolates were closely clustered together forming a distinct clade composed of two sister clades (Fig. 3, 4). The pitcher plant clade is separate from the rest of the mycobacteria species. The pitcher plant sister clades are demarcated largely by collection site, that is, isolates from LEC form a node separate of isolates from Repose Lake (Fig. 3, 4). The only isolate to not conform to this trend is DL742, which was collected from the LEC but clusters with Repose Lake samples. To determine if the pitcher plant isolates were related to *M. minnesotense*, a separate neighbor-joining tree was constructed. *M. minnesotense* was most closely related to *M. arupense* in the concatamer, which is identical to previous findings, lending reliability to the analysis [38]. The pitcher plant isolates were far removed from *M. minnesotense* indicating they were not the same species (Fig. 4). Furthermore, all preliminary trees comparing the *hsp65* of previously collected bog isolates with the current pitcher plant isolates revealed distinct clades separate of each other (Fig. A1).

2.4 Discussion

Unidentified mycobacteria were isolated from two Minnesota sphagnum bogs, the LEC and Repose Lake region, through culture and genetic analysis. Recent studies have indicated genetic analysis of mycobacteria cannot confidently be done using only one gene sequence because of the high genetic similarity seen between different mycobacteria species [50,51]. Therefore, four genes were used in this study to increase the confidence in our identification. *Hsp65* encodes for a 65kDa prokaryotic heat shock protein, *secA1* codes for a preprotein translocating ATPase, *rpoB* encodes for the RNA polymerase β subunit, and *dnaJ* codes for a hsp40-family protein possibly involved in regulation of heat-induced proteins [45–48]. *Hsp65*, *secA1* and *rpoB* are considered housekeeping genes and will positively identify species belonging to the *Mycobacterium* genus. However, these genes provide poor discrimination on a species level. Therefore, *dnaJ* is used to increase species-level robustness. *DnaJ* is a mycobacteria specific polymorphic gene with a hypervariable region that provides reliable intraspecies identification when used in conjunction with housekeeping genes [45]. While sequencing *16S rRNA* is beneficial and standard practice in microbial identification, it was explicitly not sequenced here because of its excessively weak species-level identification of mycobacteria [48,51,52]. *16S rRNA* is reliable for taxa identification, but would provide very little additional information to this study because of the use of the other housekeeping genes.

Sequence analysis indicated 18 acid-fast isolates from pitchers belonged to the *Mycobacterium* genus. All sequences were highly homologous with each other indicating low genetic diversity. This also indicates several of the isolates could be the same species. Furthermore, when comparing BLAST searches for homology, all isolates generally identify with the same set of type strains. While the isolates were positively identified as mycobacteria, the percent similarities were remarkably low and varied. For instance, DL751 was only 77% similar to *M. branderi* when analyzing the *dnaJ* gene. At the same time, the *hsp65* gene of DL751 was 96% identical to *M. mageritense.* The inconsistent species identifications and wide range in percent similarities indicate the need to use multiple genes in bacterial identification. However, conclusions should

remain conserved as sequence availability for all *Mycobacterium* species is limited. For instance, the *dnaJ* sequence for *M. mageritense* has not been published, therefore if it had been available in the BLAST databank then it could have presented as a highly similar sequence to DL751. While it remains beneficial to sequence multiple different genes, available gene sequences for reference strains should always be checked and relationships verified by phylogenetic analysis. Some genes could not amplify in all isolates, notably *secA1* and *dnaJ* (Table 2). Since the *secA1* amplicon is a large sequence its stability in the PCR process could be decreased causing it to degrade. *dnaJ* is a hypervariable gene, therefore the primer sequence may have not been homologous with the *dnaJ* of the isolates. This would suggest the isolates with no *dnaJ* amplification are more variant from the main set. Nonetheless all isolates were homologous to mycobacteria species via the *rpoB* gene, the most conserved gene to be amplified in the isolates.

Gene sequences were used to construct phylogenies by the multilocus sequence analysis (MLSA) approach. The main advantage of MLSA is the ability to construct phylogenetic trees with high resolution at the genus and species level by combining multiple different genes to form a single artificial sequence [51,53]. The total length of constructed concatamers for trees without *M. minnesotense* was 1081 bp and 748 bp with *M. minnesotense*. The neighbor-joining (NJ) and maximum likelihood (ML) with bootstrapping methods were used to construct prerequisite phylogenetic relationships. All trees with concatenates had more discriminating power than individual trees. Comparison of the NJ and ML trees were found to be similar overall, but robustness as measured by bootstrapping was higher in NJ analyses.

NJ trees indicated the pitcher plant isolates were not related to previously characterized *M. minnesotense*. This was important to determine in order to continue my investigations. If the pitcher plant isolates were indeed related to *M. minnesotense* then it would be required to determine if they were in fact *M. minnesotense*. Furthermore, all preliminary trees comparing *hsp65* of previously collected bog isolates with the current pitcher plant isolates revealed distinct clades separate of each other. Two possibilities could account

for these findings. The first possibility is that because the bog and pitcher plant samples were collected one year apart, different species were present at different times. This would indicate that at least some mycobacteria are only transient inhabitants of either the bogs or the pitcher plants and disperse by an unknown method. The second possibility is that mycobacteria isolated from pitcher plants were not inhabitants of the immediate surrounding bog, which raises the question of how mycobacteria enter the pitchers. With this in mind, several other possibilities for mycobacteria transplantation arise. Mycobacteria can be carried by aerosolized particles [54], water systems [43,55,56], and even have been found in aquatic insect salivary glands [57]. The complex cell wall of mycobacteria allow them to remain aerosolized in water droplets for long periods of time, which then could travel by wind. Bogs are waterlogged so water transport is unlikely, but aquatic insects are common in pitcher plants making insects a strong possibility for mycobacteria transport. In order to determine the mode of transmission in Minnesota, more experiments will be needed to confirm these findings.

Phylogenetic trees revealed the pitcher plant isolates formed a unique clade separate from all reference strains (Figs. 3, 4). The split between pitcher plant isolates and reference species in all trees was reliable as the bootstrap values were always 90% or higher. The closest neighbor was *M. chelonae,* however it was not indicated as being genetically similar via BLAST searches for any of the four genes, of which all are available for *M. chelonae.* To confirm it was not *M. chelonae,* it is well documented that *M. chelonae* is nonpigmented, and produces varied colonies between 25°C and 40°C [58]. As decribed in Part II, the pitcher plant isolates do not resemble this morphologic profile. Therefore, the next closest neighbors *M. flavescens, M. mucogenicum,* and *M. vaccae* were further studied. Within the pitcher plant clade, two sister clades emerge based on collection site, with DL742 being the only variant. This trend reveals large-scale bacterial species variation between pitcher plants in different bogs, which coincides with previous studies on bacterial assemblages in pitcher plants [39]. Small scale variation, between pitchers within the same bog, was not significant. Resolution within the pitcher plant clade was poor, as indicated by the short branches, therefore it is likely several isolates are the same

species or variants of each other. It is unclear whether the pitcher plant selects for a narrow range of mycobacteria or there exists rapid evolution of mycobacteria to survive within the pitcher plant after seeding.

Because of the generally low sequence similarities and separation into two sister clades, it was proposed two novel mycobacteria species were identified in the pitcher plants. Since some genes could not amplify in all isolates with the primers used, metabolic and growth tests were conducted in Chapter 3 to characterize the isolates and better determine their speciation and type strain.

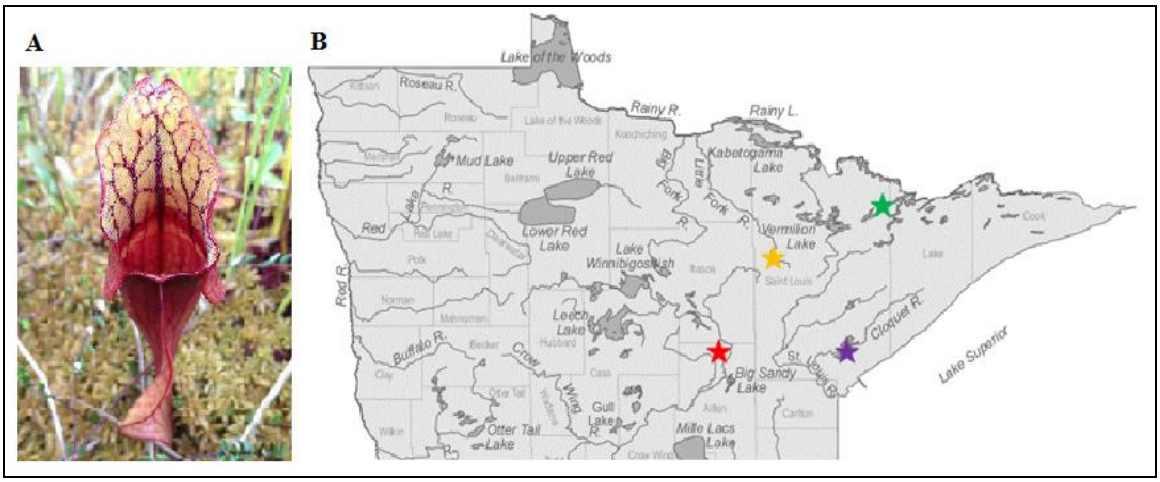

Figure 1. Collection schematic. A, Mature *Sarracenia pupurea* pitcher. **B,** All pitcher plant water samples were collected in northern Minnesota between 2012 and 2013. Green star, Repose Lake collected in August 2012; yellow star, Laurentian Environmental Center collected in June 2013; purple Star, Duluth collected in July 2013; red star, Savanna Portage State Park collected in October 2013.

Table 1. Primers used for the amplification and sequencing of genes *dnaJ, hsp65, secA1,* and *rpoB*. All positions and names are as stated in the original publication.

Primer Name	Sequence (5' → 3')	Target Gene	Position	Reference
J10F	CGIGARTGGGTYGARAARG	*dnaJ1*	10[a]	Yamada-Noda *et al*., 2007
J335R	ARICCICCGAAIARRTCICC	*dnaJ1*	376[a]	Yamada Noda *et al*., 2007
Tb11	TGTAAAACGACGGCCAGTACCAACGATGGTGTGTCCAT	*hsp65*	396[b]	Ringuet *et al*., 1999
Tb12	CAGGAAACAGCTATGACCCTTGTCGAACCGCATACCCT	*hsp65*	836[b]	Ringuet *et al*., 1999
Mtu.Forward 1	GACAGYGAGTGGATGGGYCGSGTGCACCG	*secA1*	412-440[c]	Zelazny *et al*., 2005
Mtu.Reverse 3	ACCACGCCCAGCTTGTAGATCTCGTGCAGCTC	*secA1*	1141-1172[c]	Zelazny *et al*., 2005
MycoF	GGCAAGGTCACCCCGAAGGG	*rpoB*	2573-2592	Adekambi *et al*., 2003
MycoR	AGCGGCTGCTGGGTGATCATC	*rpoB*	3316-3337	Adekambi *et al*., 2003

[a] Position relative to the *Mycobacterium tuberculosis dnaJ1* sequence.

[b] Position relative to the *Mycobacterium tuberculosis hsp65* sequence.

[c] Position relative to the *Mycobacterium tuberculosis secA1* sequence.

Table 2. Homology search results for *rpoB, hsp65, secA,* and *dnaJ* genes determined by BLAST. First and second highest similarities are reported in percentage of the total query sequence. NA=not amplified by the primers used here. IC=incomplete; the sequence was amplified by the primers, but failed to isolate and sequence.

Isolates	*rpoB* gene		*hsp65* gene		*secA* gene		*dnaJ* gene	
	First and second matches (%)		First and second matches (%)		First and second matches (%)		First and second matches (%)	
DL725	*M. rhodesiae* (94)	M. sp. ATCC 21498 (94)	*M. mageritense* (95)	M. sp. FL04-42-158A (95)	*M. brumae* (94)	*M. canariasense* (94)	*M. flavescens* (83)	*M. vaccae* (82)
DL726	*M. rhodesiae* (95)	M. sp. ATCC 25793 (95)	*M. mageritense* (96)	M. sp. FL04-42-158A (95)	*M. canariasense* (92)	*M. septicum* (91)	*M. flavescens* (83)	*M. vaccae* (82)
DL727	*M. canariasense* (91)	*M. pallens* (91)	NA		NA		NA	
DL728	*M. rhodesiae* (94)	M. sp. ATCC 21498 (94)	*M. mageritense* (96)	M. sp. FL04-42-158A (95)	*M. canariasense* (91)	*M. perigrinum* (90)	*M. flavescens* (82)	*M. vaccae* (82)
DL729	*M. rhodesiae* (93)	M. sp. ATCC 21498 (92)	*M. mageritense* (96)	M. sp. FL04-42-158A (95)	*M. flavescens* (91)	*M. fortuitum* (91)	*M. flavescens* (83)	*M. vaccae* (82)
DL730	*M. rhodesiae* (93)	M. sp. ATCC 21498 (92)	*M. mageritense* (96)	M. sp. FL04-42-158A (95)	*M. flavescens* (91)	*M. fortuitum* (91)	*M. flavescens* (83)	*M. vaccae* (82)
DL731	*M. rhodesiae* (93)	M. sp. ATCC 21498 (92)	*M. mageritense* (96)	M. sp. FL04-42-158A (95)	*M. flavescens* (91)	*M. gordonae* (91)	*M. flavescens* (82)	*M. nonchromogenicum* (76)
DL732	M. sp. AFP-00017 (91)	*M. canariasense* (91)	*M. mageritense* (96)	M. sp. FL04-42-158A (95)	*M. canariasense* (92)	*M. fortuitum* (92)	*M. flavescens* (82)	*M. vaccae* (81)
DL733	*M. rhodesiae* (94)	*M. lacticola* (93)	*M. mageritense* (96)	M. sp. FL04-42-158A (95)	NA		NA	
DL734	*M. rhodesiae* (92)	M. sp. ATCC 21498 (92)	*M. mageritense* (96)	M. sp. FL04-42-158A (95)	*M. canariasense* (93)	*M. brumae* (93)	*M. flavescens* (82)	*M. scrofulaceum* (79)

Table 2 continued.

Isolates	*rpoB* gene		*hsp65* gene		*secA* gene		*dnaJ* gene	
	First and second matches (%)		First and second matches (%)		First and second matches (%)		First and second matches (%)	
DL735	*M. rhodesiae* (92)	M. sp. ATCC 21498 (92)	*M. mageritense* (96)	M. sp. FL04-42-158A (95)	*M. canariasense* (94)	*M. brumae* (93)	NA	
DL736	*M. rhodesiae* (92)	M. sp. ATCC 21498 (92)	*M. mageritense* (96)	M. sp. FL04-42-158A (95)	NA		NA	
DL737	*M. rhodesiae* (92)	M. sp. ATCC 21498 (92)	*M. mageritense* (96)	M. sp. FL04-42-158A (95)	*M. canariasense* (91)	*M. septicum* (90)	IC	
DL738	*M. rhodesiae* (95)	M. sp. ATCC 21498 (95)	M. sp. ATCC 21498 (96)	M. sp. WR88 (96)	*M. gordonae* (91)	*M. asiaticum* (91)	*M. cookii* (80)	*M. branderi* (77)
DL739	*M. rhodesiae* (95)	M. sp. ATCC 21498 (95)	M. sp. ATCC 21498 (96)	*M. aichiense* (95)	*M. peregrinum* (91)	*M. gordonae* (91)	*M. cookii* (80)	*M. branderi* (78)
DL742	*M. rhodesiae* (93)	M. sp. ATCC 21498 (92)	*M. mageritense* (96)	M. sp. FL04-42-158A (95)	*M. flavescens* (90)	*M. gordonae* (90)	*M. scrofulaceum* (81)	*M. simiae* (78)
DL750	*M. rhodesiae* (93)	M. sp. ATCC 21498 (95)	NA		*M. gordonae* (90)	*M. peregrinum* (90)	*M. branderi* (80)	*M. hiberniae* (80)
DL751	M. sp. ATCC 21498 (95)	*M. rhodesiae* (95)	M. sp. ATCC 21498 (96)	M. sp. WR88 (96)	IC		*M. cookii* (80)	*M. branderi* (77)

- ATCC 21498 and ATCC 25793 are also *M. petroleophilum* and *M. chlorophenolicum*, respectively, in the American Tissue Culture Collection databank.

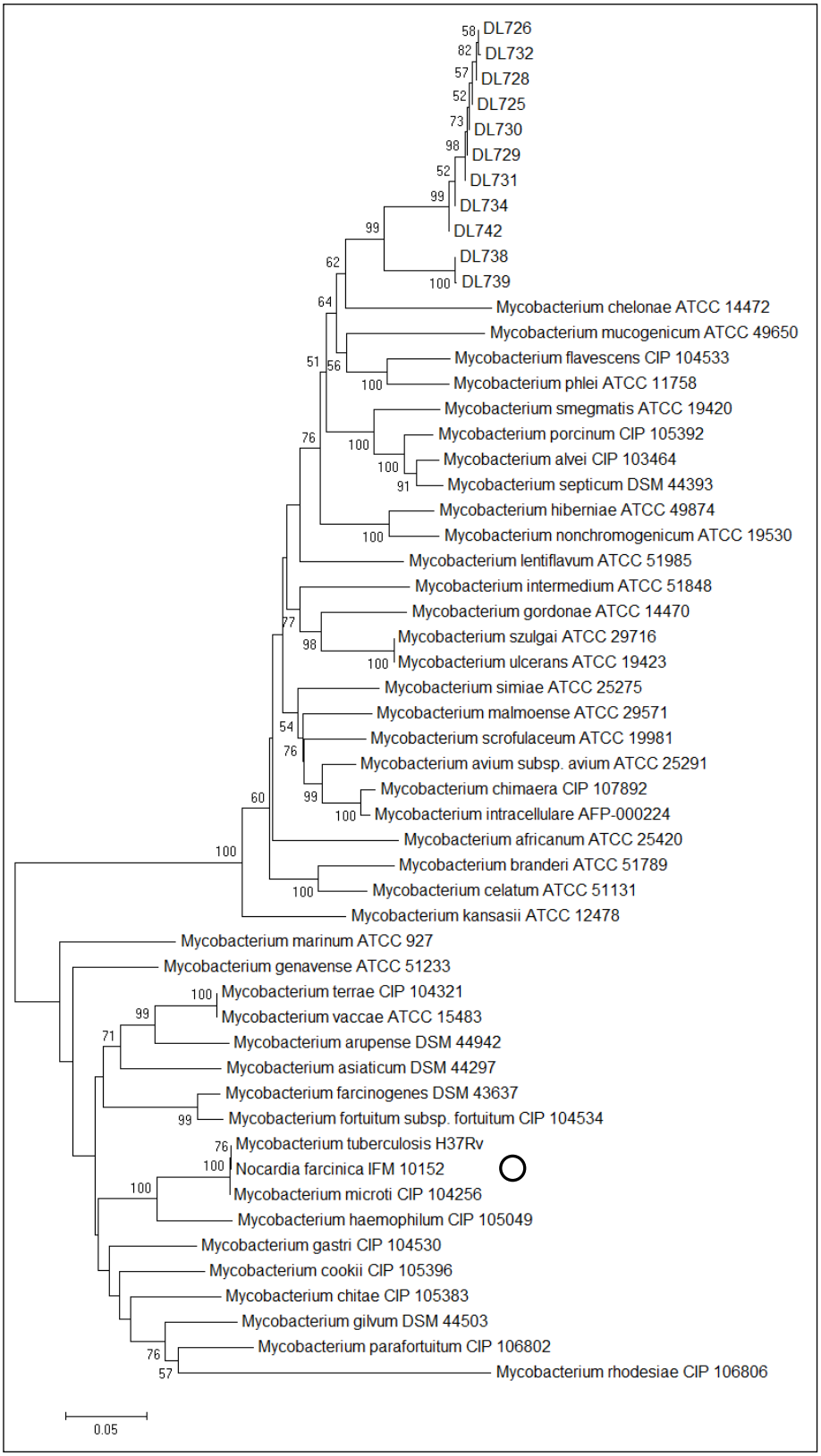

Figure 2. Analysis of *Nocardia farcinica* as a suitable outlier. The evolutionary history was inferred by using the Neighbor-Joining method with the concatamer nucleotide sequence *rpoB-dnaJ-hsp65*. The total length was 1196 bp. The evolutionary distances were computed using the Kimura 2-parameter method with units of number of base substitutions per site. The outlier was *N. farcinica* IFM 10152 (indicated by the open circle) although no outlying group was indicated in the tree. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches with a 50% cutoff.

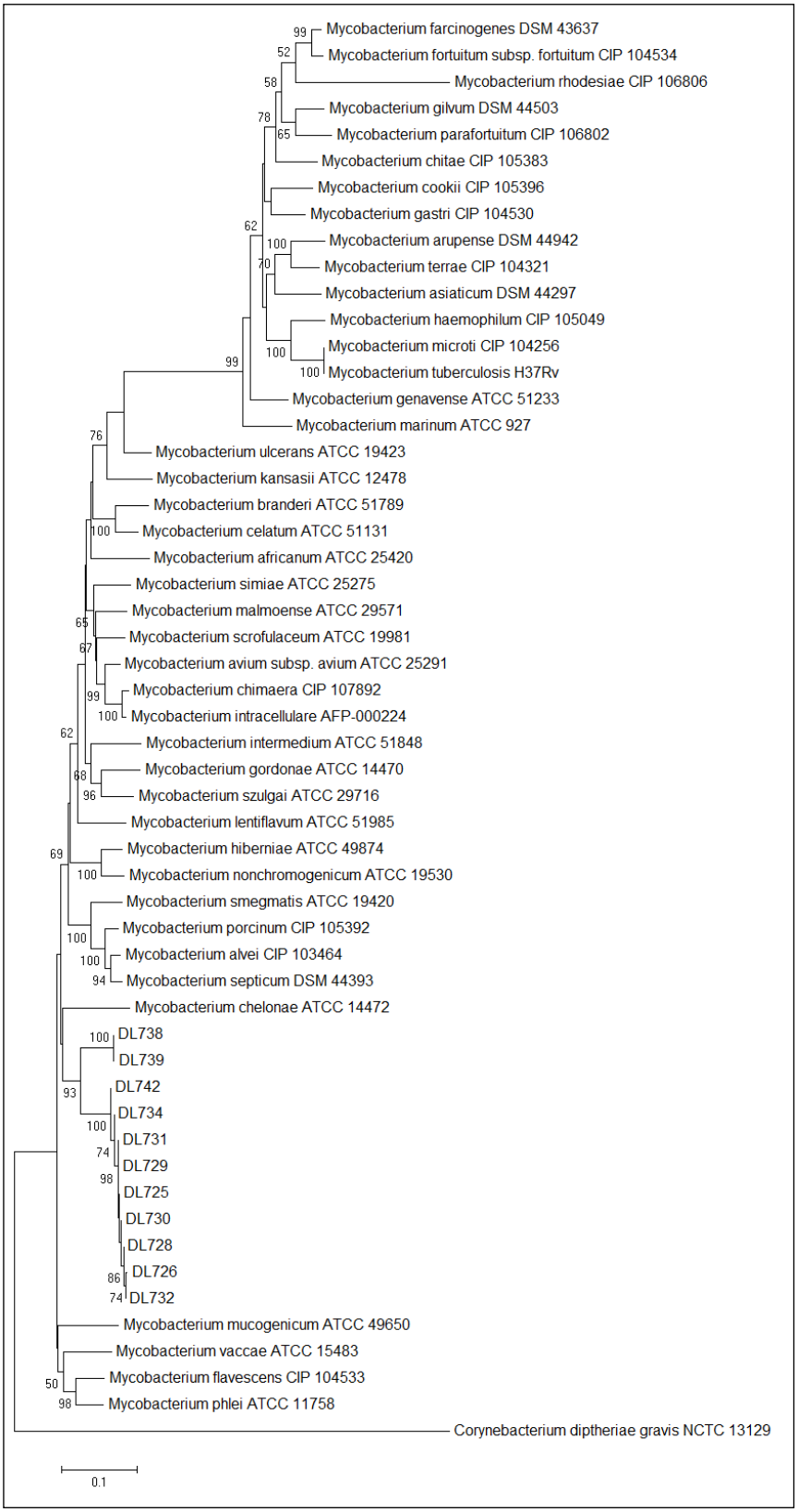

Figure 3. Evolutionary relationship of pitcher plant isolates and accepted *Mycobacterium* species. The evolutionary history was inferred by using the Neighbor-Joining method with the concatamer nucleotide sequence *rpoB-dnaJ-hsp65*. The total length was 1081 bp. The evolutionary distances were computed using the Kimura 2-parameter method with units of number of base substitutions per site. The tree was rooted using *Corynebacterium diptheriae gravis* NCTC 13129. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches with a 50% cutoff.

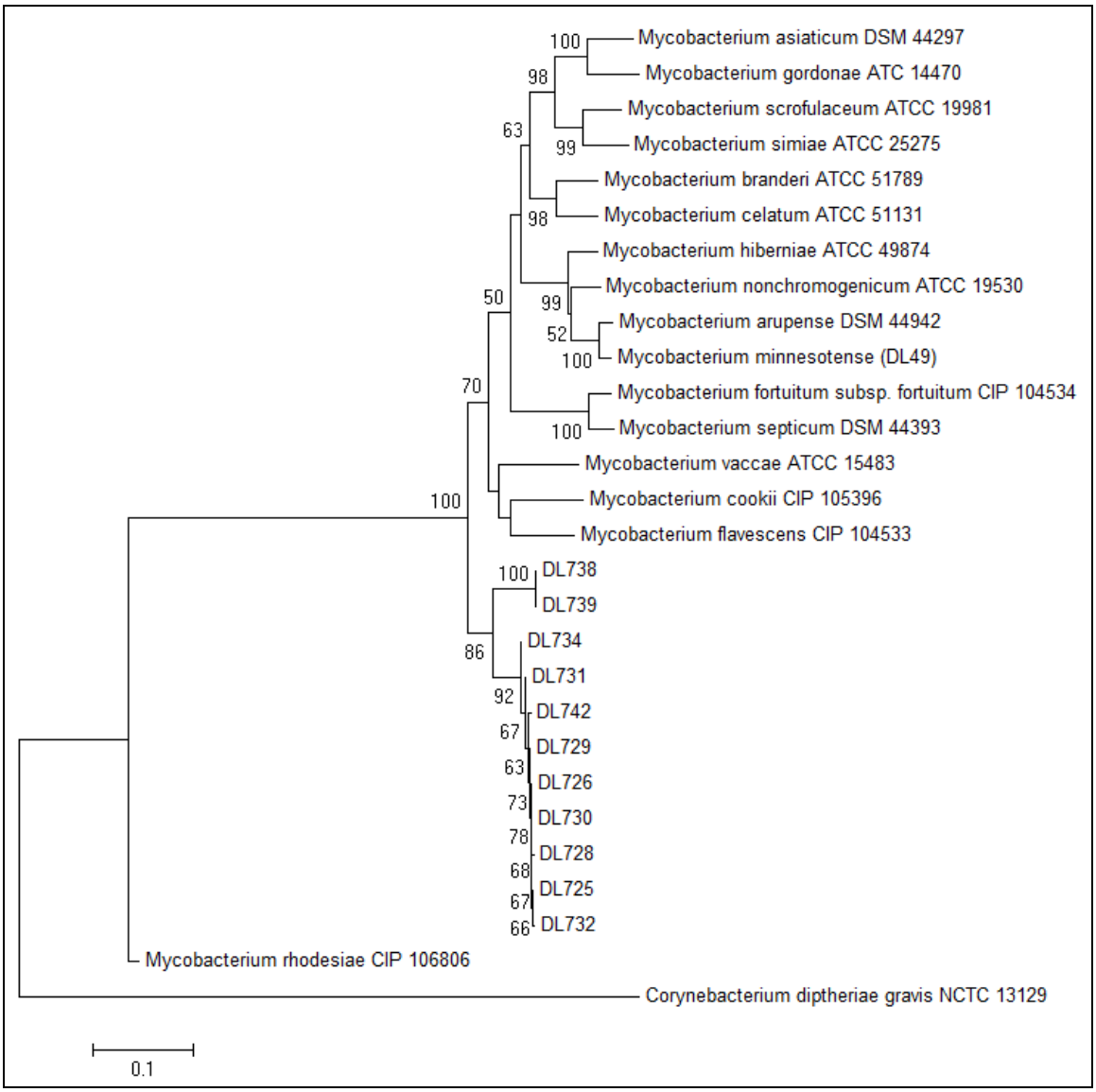

Figure 4. Phylogenetic analysis of pitcher plant isolates, *M. minnesotense,* and most similar mycobacteria species as determined by BLAST. The evolutionary history was inferred by using the Neighbor-Joining method with the concatamer nucleotide sequence *dnaJ-hsp65*. The total length was 748 bp. The evolutionary distances were computed using the Kimura 2-parameter method with units of number of base substitutions per site. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches with a 50% cutoff. The tree was rooted using *Corynebacterium diptheriae gravis* NCTC 13129 and all *Mycobacterium* species from Table 1 that had sequences for *dnaJ* and *hsp65*.

Chapter 3: Characterization of Isolates

3.1 Introduction

One of the main objectives of this work was to identify and analyze mycobacteria that potentially reside in *S. purpurea* pitchers. As with all organisms, bacteria have predictable behaviors that can be used to characterize and distinguish species from each other. Bacteria can be categorized based on multiple factors including optimal growth parameters, carbon and nitrogen utilization, antibiotic susceptibility, enzyme production, and cellular structure. This chapter will focus on characterizing the mycobacteria isolates identified in Part I.

All cellular life requires a plasma membrane to contain and separate itself from its environment. Most plasma membranes are heterogeneously composed of diverse lipid species and membrane-embedded proteins. Eukaryotes have a single cell membrane surrounding many bound organelles whereas most bacteria contain a single plasma membrane protected by a cell wall. On the most basic level, bacteria can be either Gram-negative or Gram-positive depending on the peptidoglycan-based membrane. Gram-positive bacteria are those that synthesize a single layered membrane composed of a thick layer of peptidoglycan. In contrast, Gram-negative bacteria possess a thin layer of peptidoglycan in between two phospholipid bilayers. Mycobacteria are traditionally classified as Gram-positive because of their thick cell wall, but do not technically belong to either category. Instead, they have evolved a specialized dual membrane system that consequently lends to their innate resistance to drugs, disinfectants and biocides [6,7].

The mycobacteria cell wall contains an inner phospholipid plasma membrane with embedded membrane proteins. The plasma membrane is separated from the outer membrane by the periplasm space [59]. Innate resistance is attributed to the hydrophobic outer membrane composed of long chain mycolic acids that are covalently bound to arabinogalactan-peptidoglycan polymers [6]. Mycolic acid has two carbon tails (up to 90 carbons long) that become chemically modified. Every mycobacteria species modifies its mycolic acids slightly differently, thus creating species-specific mycolic acid profiles that

can be analyzed by gas chromatography to identify different species [6]. Bacterial species can also be identified by phospholipid profiles since lipid species composition is constant for any given organism. These sensitive chemotaxonomic analyses are new standards that can precisely locate a species taxonomic position.

The thick mycolic acid cell wall strongly influences mycobacteria's slow replication time. The synthesis of numerous long-chain fatty acids requires large amounts of energy and carbon, therefore division rates are slower. In the bacteria kingdom, mycobacteria are among some of the slowest growing organisms with slow-growing species requiring up to a month to form a visible colony in the lab. Fast-growing mycobacteria require less than seven days to form a visible colony, still slower than most other bacteria such as *Escherichia coli* or *Bacillus* species. Therefore, growth rate can be a distinguishing factor of a bacterial species.

Analyses that identify enzyme production can be used to create biochemical profiles. Bacteria only produce enzymes required to survive in a given environment, therefore enzyme species will vary depending on the bacteria, even within a genus. For instance, arylsulfatase, an enzyme that hydrolyzes bonds in compounds with aromatic rings, is exclusively attributed to mycobacteria. Slow-growing mycobacteria, such as *M. marinum*, produce arylsulfatase after 14 days whereas fast-growing mycobacteria, such as *M. fortuitum*, produce arylsulfatase after only 3 days. Even with the advent of genomics and molecular-based identification techniques, biochemical profiles continue to play cardinal roles in bacterial identification. Antibiotic resistance and susceptibility can also be used to identify and distinguish specific strains. Genes for antibiotic resistance are often transferred horizontally on plasmids between different bacteria. In order for horizontal transfer to occur, organisms are required to inhabit the same environment for a given amount of time. Thus bacteria with similar antibiotic resistance profiles are likely to be genetically similar and have shared an environment.

In Chapter 3 I have characterized the pitcher plant isolates based on metabolic growth properties, fatty acid analysis, biochemical analysis, antibiotic sensitivity, and biofilm

formation as a means to precisely identify the mycobacterial species. The characterization provides evidence and support for the previous phylogenetic analyses in that the closest evolutionary neighbors are phenotypically and chemically different from the pitcher plant isolates. As with all bacteria, there exists natural variation in these features. Therefore, all isolates were used to create an average, typical description and to aid in identifying the type strains. It was determined that two different species were present and the type strains DL734 and DL739 were chosen to represent these two species. DL734 represents strains DL725-DL737 and DL742, while DL739 represents DL738, DL739, DL750, and DL751.

3.2 Materials and Methods

3.2.1 Materials and Statistical Analysis

All Middlebrook 7H9 media in this chapter was supplemented with 10% albumin dextrose catalase (ADC) (v/v), 0.05% Tween 80 (v/v) and 0.2% glycerol (v/v). All Middlebrook 7H11 media was supplemented with 10% oleic albumin dextrose catalase (OADC) (v/v) and 0.2% glycerol. These will be referred to as 7H9 and 7H11, respectively.

All statistical significance tests compared means for DL734 strains and DL739 strains using the Students two-tailed *t* test assuming unequal variances and a *P* value of <0.05 was considered to show a significant difference.

3.2.2 Metabolic Growth Properties

All pitcher plant samples were grown on 7H11 plates at 28°C, 32°C or 37°C. Growth characteristics including colony morphology, color, size, and growth rate were consequently examined 5 days, 7 days, and two weeks after growth initiation. The isolates are then compared to different reference strains to determine which mycobacteria species they are most related to. The reference strains included those that are the closest

neighbors in the phylogenetic analysis and any other strain that appeared in the BLAST homology searches.

3.2.3 Scanning Electron Microscopy (SEM)

SEM was used to determine cellular morphology. Samples were prepared by modifying the Innovotech MBEC HTP assay procedure [60]. 10 µL aliquots of each isolate was placed on a glass cover slip and allowed to air dry for 48 hrs. Cover slip samples were fixed using 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer, pH=7.4, for 16 hrs at 4°C. Afterwards, samples were washed in the fixative for 10 min then washed with dH_2O for 5-10 min. Samples were dehydrated in 70% ethanol for 20 min at room temperature, rinsed with dH_2O, then air dried for 24 hrs. The sample was mounted onto an aluminum sample stub with conductive tape, then sputter coated with approximately 6 nm of gold using a Denton Vacuum Desk IV. A JEOL JSM-6490LV scanning electron microscope was used to capture secondary electron images at an accelerating voltage of 10 kV.

3.2.4 Growth Curves

Colonies were swabbed into 5 mL 7H9 broth and incubated overnight with shaking at 28°C and 32°C. Samples were left to settle and the supernatant was removed and added to 15 mL 7H9 broth assuming the supernatant was a monosuspended layer. Solutions were equilibrated to an OD_{600} of 0.1 with a final volume of 30 mL in Erlenmeyer flasks using a Cole Parmer 1100 Spectrophotometer. Flasks were covered in tin foil for aeration. Solutions were incubated with shaking at 28°C or 32°C. OD_{600} readings were recorded every 4-6 hours for up to 5 days. All samples were run in duplicate then averaged.

3.2.5 Antibiotic Sensitivity

Each pitcher plant isolate was swabbed onto 7H11 plates. *M. gordonae* was the control. Antibiotic disks were placed in the center of each plate which were then incubated for 7 days. Because there are no standards to determine resistance or susceptibility for nonpathogenic mycobacteria, zones of inhibition were measured to indicate relative

sensitivity to the antibiotics. Antibiotics tested were (µg per disk unless otherwise stated): ampicillin (10), aztreonam (30), chloramphenicol (30), ciprofloxacin (5), neomycin (30), penicillin (10), streptomycin (10), tetracycline (30), and trimethoprim (30). To determine significance, zones of inhibitions were pooled and averaged to compare DL734 associated strains and DL739 associated strains.

3.2.6 Fatty Acid Methyl Esters Analysis (FAME)

FAMEs were obtained for 12 of the pitcher isolates and for *M. gordonae* by saponification, methylation, and extraction. FAME profiles were then separated by gas chromatography, and identified using the Sherlock Microbial Identification System (MIDI, Inc.). Samples were grown on solid media then sent to Microbial ID, Inc. (Newark, DE) for FAME analysis.

3.2.7 Biochemical Properties

All environmental isolates were subjected to the following biochemical tests, as previously described [61,62]: Tween 80 hydrolysis with *M. kansasii* as the control, urease hydrolysis with *M. scrofulaceum* as the control, nitrate reductase with *M. fortuitum* as the control, tellurite reduction with *M. avium* as the control, 3 and 14-day arylsulfatase with *M. smegmatis* as the 3-day and *M. fortuitum* as the 14-day controls, and 5% NaCl tolerance with *M. gordonae* as the control. Additionally, the heat stable 68°C catalase test was performed with *M. gordonae* as the control, as previously described [63].

3.2.8 Biofilm Formation

100 µL of 7 day old liquid cultures were used to inoculate 5-mL aliquots of 7H9 in glass tubes. Samples were incubated at room temperature for 2 months without shaking and visually analyzed for biofilm formation.

3.3 Results

3.3.1 Metabolism and Morphology

The pitcher plant isolates were bent rod shaped, and exhibited fast to intermediate growing colonies with optimal growth temperatures between 28-32°C (Fig. 5). DL738, DL739, DL750, and DL751 from 28°C to 37°C with 37°C being the optimal growth temperature for DL739 (Table 3). All isolates were scrotochromogenic with some exhibiting varying levels of photochromogenicity. In other words, all isolates produced pigment regardless of light exposure and pigment levels increased with light exposure in photochromogenic species. Colony morphology showed variation with most isolates producing smooth, convex colonies (Fig. 6). When compared to morphology profiles of other mycobacteria species, the pitcher plant isolates were dissimilar to their closest neighbors *M. mucogenicum*, *M. cookii*, *M. flavescens*, and *M. vaccae*. *M. mucogenicum* is a cream colored, fast growing species, *M. cookii* is slow growing and replicates at 37°C, *M. flavescens* is yellow-cream colored forming distinctively rugged and dry colonies, and *M. vaccae* is photochromogenic. In fact, they are morphologically most similar to a more distant neighbor that was infrequently identified by BLAST homology searches–*M. gordonae*. *M. gordonae* was scrotochromogenic with increased pigmentation when exposed to light, replicates between 28°C and 37°C, and produces smooth convex colonies. Thus *M. gordonae* was chosen as the reference strain in further studies. DL734 was chosen as the type strain for the Repose Lake isolates, DL737, and DL742. DL739 represented the remaining LEC isolates: DL738, DL750 and DL751. Unless otherwise stated, DL734 and DL739 will be inclusive of all strains tested.

3.3.2 Growth Curves

Growth curves for all pitcher plant isolates, *M. gordonae*, *M. flavescens*, and *M. vaccae* were conducted at 28°C and 32°C over 5 days. DL734 and DL739 strains showed significantly different replication rates at 28°C ($t = 2.56$, $P = 0.02$) and at 32°C ($t = 4.38$, $P = 2.6\text{x}10^{-4}$). All samples showed robust growth in liquid, except for *M. vaccae* which failed to replicate (Figs. 7, 8). At 28°C, all samples reached mid-log phase at ~60 hrs and

the curve patterns more closely resembled *M. gordonae* (Fig. 7). At 32°C, DL739 reached mid-log phase at ~50 hrs while DL734 reached mid-log phase at 60 hrs (Fig. 8). DL734 did not replicate at 32°C and the associated strains only reached half their maximum cell density values when compared to 28°C growth. The growth pattern exhibited by DL734 resembled *M. gordonae* at both temperatures with *P* values > 0.05 (Figs. 7, 8). In contrast, DL739 grew to similar maximum cell density values regardless of temperature, but reached this point at a faster rate when incubated at 32°C. DL739 exhibited growth curve patterns markedly similar to *M. flavescens* at 32°C, but dissimilar to all reference strains at 28°C (Figs. 7, 8). Again geographic differences are evident in the growth curves with LEC isolates surviving better than Repose Lake isolates. The growth patterns of DL737 and DL742 further validates its categorization with DL734. Refer to figures A2 and A3 in the appendix for growth curves of all strains.

3.3.3 Antibiotic Sensitivity

Pitcher plant isolates and *M. gordonae* were subjected to antibiotic sensitivity testing using antibiotic-laden disks on agar plates. The antibiotics were divided into three general categories: those that inhibited protein synthesis (chloramphenicol, neomycin, streptomycin, and tetracycline), those that are enzymatic inhibitors (ciprofloxacin, and trimethoprim), and those that affected cell wall synthesis (ampicillin, aztreonam, and penicillin). *M. gordonae* was sensitive to only chloramphenicol and ciprofloxacin with zones of inhibition of 15 mm and 30 mm, respectively (Fig. 9, B, C). All isolates were resistant to aztreonam and trimethoprim, and susceptible to neomycin and tetracycline (Fig. 9, D, G). DL734 was susceptible to ampicillin, chloramphenicol, ciprofloxacin, streptomycin, and penicillin (Fig. 9, A-C, F, E). Exceptions were DL737 and DL742 which are resistant to penicillin (Fig. 9, E). DL739 was susceptible to chloramphenicol, ciprofloxacin, and streptomycin, and resistant to penicillin (Fig. 9, B, C, F, E). To distinguish strain DL734 from strain DL739, there was significant difference in the mean zone of inhibitions for ampicillin ($t = 7.48$, $P = 1.96 \times 10^{-6}$), chloramphenicol ($t = 8.35$, $P = 5.03 \times 10^{-7}$), ciprofloxacin ($t = 4.45$, $P = 0.021$), penicillin ($t = 5.58$, $P = 1.2 \times 10^{-4}$),

streptomycin (t = 5.25, P = 1.58x10^{-4}), and tetracycline (t = 4.35, P = 7.81x10^{-4}). However, there was no difference in neomycin sensitivity.

3.3.4 Fatty Acid Methyl Ester Analysis

FAME profiles are routinely used to identify bacteria species based on phospholipid species present in the cell membranes. FAME analysis is highly sensitive and can often distinguish between two species that are nearly morphologically and genetically identical. FAME analysis was done on 12 of the isolates at Microbial ID, Inc. These were chosen to represent the entire collection with *M. gordonae* as the reference profile. The FAME profile for *M. gordonae* was correctly identified, but the pitcher plant isolates were unidentified within the *Mycobacterium* genus. All isolates showed variation in their fatty acid lipid profiles, but are more constant with each other than with *M. gordonae* (Table 4). With the exception of *M. gordonae*, the majority of the isolates produced peaks $C_{10:0}$ (decanoic acid), $C_{17:1}$*w7c* ((10Z)-10-Heptadecenoic acid) and 10Me-$C_{18:0}$ TBSA (10-Methyloctadecanoic tuberculostearic acid) (Table 4, Fig. 10). *M. gordonae* is the only profile to exhibit production of 2-Me-$C_{14:0}$ (2-Methyltetradecanoic acid), $C_{17:0}$ (Heptadecanoic acid), and $C_{17:1}$*w8c* ((9Z)-9-Heptadecenoic acid) (Table 4). DL739 strains were the only ones to produce 8-Me-$C_{16:0}$ (8-Methylhexadecanoic acid) and $C_{20:0}$ (icosanoic acid) (Fig. 10). All DL739 isolates and two DL734 isolates produced $C_{20:0}$ ALC 18.838 ECL (Table 4, Fig. 10). This would indicate that DL734 and DL739 are more closely related to each other than to *M. gordonae.* Representative chromatographs showed that while the majority of lipids produced were the same for *M. gordonae* and the pitcher plant isolates, quantities of these lipids varied (Fig. 10). Please refer to figures A4-A15 in the appendix for the complete set of FAME chromatographs.

3.3.5 Biochemical Properties

Follows is a summary of the biochemical properties for the type strains DL734, DL739, *M. vaccae, M. flavescens,* and *M. gordonae.* Results for all individual strains are recorded in Table 3.

M. gordonae reduced nitrate and produced arylsulfatase after 3 and 14 days. It did not tolerate NaCl at 5%, hydrolyze Tween 80 or urea, reduce tellurite, or produce catalase. *M. vaccae* test results were similar with the following exceptions: it minimally produced urease, and produced catalase. *M. flavescens* was positive for Tween 80 and urease hydrolysis, nitrate reduction, and catalase. It was negative for 3-day arylsulfatase and inconclusive results were obtained for 14-day arylsulfatase and tellurite reduction.

DL734 and its closely-associated pitcher isolates did not hydrolyze Tween 80, reduce tellurite, or produce urease and catalase. The isolates did not tolerate growth in the presence of 5% NaCl. DL734 tested positive for 3-day arylsulfatase (33% of these isolates were positive), 14-day arylsulfatase, and nitrate reduction (66% of these isolates were positive) (Table 3).

DL739 and its associated isolates did not produce catalase and did not reduce nitrate or tellurite. DL739 (66% of these isolates) minimally grew on 5% NaCl, hydrolyzed Tween 80, produced urease and performed 3-day arylsulfatase; all of these isolates were positive for 14-day arylsulfatase (Table 3).

3.3.6 Biofilm Formation.

All pitcher plant isolates were grown in glass culture tubes to observe the ability to form biofilms. A majority of strains did not produce biofilms within 2 months (Fig. 11). DL725, DL727, DL731, and DL751 produced biofilms to varying degrees of intensity (Fig. 11) DL731 produced a vertically spreading biofilm while DL725 and DL727 produced a biofilm that remained at the air-water interface. Of interesting note, DL728, DL729, DL732, DL734, and DL735 produced strong biofilms when grown in plastic conical tubes, but not in glass tubes (data not shown). This affect could be due to multiple reasons including different substrate preference or a lower initial cell density in the glass tube experiments. This would indicate a preference for certain substrates or the existence of a critical cell density to begin biofilm formation.

3.4 Discussion

Bacterial morphology and certain biochemical properties remain consistent over time and are unique among different species making it a powerful discriminating tool. When taken as a whole, the biochemical profiles of bacteria are minutely different enough to distinguish one bacterium from another. However, it is important to recognize that natural variation through evolution exists between colonies of the same species, which is why all pitcher plant isolates and *M. gordonae* were examined in these studies. The biochemical evidence suggests there are two distinct bacterial strains represented in the pitcher plant isolates, thus the need for two separate type strains. DL734 represents all strains from Repose Lake (DL725-DL736), DL737, and DL742, while DL739 represents DL738, DL750, and DL751. As can be seen in any given test, DL734 and DL739 are not representative of all its constituent strains, however by having data for all isolates an average profile can be created which is most accurately captured by DL734 and DL739. In this assessment, the most typical trends will be discussed and the type strains will be the only strains referenced. The two type strains will also be the only ones submitted for depository in the Japan Collection of Microorganisms and the Netherlands Culture Collection of Bacteria collection banks.

All pitcher plant isolates shared cell morphology and growth rates similar to known fast-growing mycobacteria species. DL734 tended to grow more optimally at 28°C than at 32°C, while DL739 exhibited similar growth regardless of temperature. DL738 and DL739 were the only ones to rapidly grow at 37°C with DL750 and DL751 exhibiting slightly decreased growth rates. As most known mycobacteria preferentially grow between 30°C and 37°C, with very few species tolerating cooler temperatures, the pitcher plant isolates preference for cooler temperatures could indicate adaptation to the bog and pitcher plant environments. Most isolates could not form biofilms in glass tubes indicating biofilms are not a crucial mode of life for mycobacteria in pitcher plants.

Based on colony morphology and growth parameters, *M. gordonae* was chosen as the reference strain and the most related M*ycobacterium* species even though it was an infrequent match genetically (see Chapter 2). This is seemingly contradictory to results

discussed in Chapter 2 where *M. flavescens*, *M. cookii,* and *M. vaccae* were indicated as the closest related neighbor to the pitcher plant isolates. However, upon further analysis of the phylogenetic relationships in Chapter 2, DL739 is branched consistently closer in relation to known mycobacteria than DL734. DL739 strains also produce colonies and growth rates more similar to *M. flavescens* and *M. vaccae* than do the DL734 strains. Therefore, DL739 could be related to *M. flavescens* or *M. vaccae* whereas DL734 is more distantly related. However, DL739 does not share a growth rate with *M. flavescens* or *M. vaccae* at 28°C which argues against it being *M. flavescens.* On the other hand, DL734 continued to exhibit growth patterns consistent to *M. gordonae* with no statistical difference. *M. gordonae* remains a reliable type strain for all isolates as it grows from 28°C to 37°C and has an intermediate growth rate. *M. gordonae* is coincidentally routinely found in sphagnum bogs [1,64].

The antibiotic profiles of the isolates suggest two different sensitivities. All isolates were resistant to aztreonam and trimethoprim, which are not antituberculosis medications. Compared to DL739, DL734 was more susceptible to the β-lactam antibiotics ampicillin and penicillin. DL739 showed complete resistance to penicillin and 50% of DL739 strains were resistant to ampicillin. This is to be expected as β-lactam antibiotics target peptidoglycan synthesis, a cell wall component found inside the mycolic acid layer in mycobacteria. Chloramphenicol, which is not used to treat tuberculosis, partially inhibited growth of DL734 and DL739. The isolates were susceptible to streptomycin, a second line tuberculosis medication, and ciprofloxacin which can be used against tuberculosis if first and second line drugs fail. Neomycin, a derivative of streptomycin produced moderate effects on growth and most likely would not be used in treatment against these isolates. *M. gordonae* was resistant to ampicillin, neomycin, streptomycin, and penicillin. This is the first report of antibiotic resistance, especially streptomycin, in *M. gordonae.* Since *M. gordonae* is not known to cause disease, clinical antibiotic tests are uncommonly requested and no standard for determining resistance vs susceptibility has been established.

Because lipid distribution and concentration varies between species, FAME provides a high resolution analysis with the ability to distinguish between very similar species. Often times it is the only distinguishing factor between two highly similar species. Lipid composition of selected isolates and *M. gordonae* indicate at least three different lipid profiles. The pitcher plant isolates were markedly different from *M. gordonae* due to production of $C_{17:1}$*w7c* and 10Me-$C_{18:0}$ TBSA. To corroborate the data, it has been previously determined that TBSA, or tuberculostearic acid, is present in different concentrations in all mycobacteria except for *M. gordonae* [65]. The pitcher plant isolates were again divided into two groups due to $C_{20:0}$ and 8-Me-$C_{16:0}$ synthesis solely by DL739. To rule out the isolates' closest phylogenetic neighbors *M. cookii* and *M. flavescens,* it was previously determined that the majority of lipids synthesized by *M. cookii* are $C_{22:0}$ and $C_{24:0}$, while *M. flavescens* produces $C_{14:0}$ and $C_{15:0}$ [66,67]. None of these lipid species are expressed by the pitcher plant isolates. Therefore, it is unlikely the pitcher plant isolates are *M. cookii* or *M. flavescens.* The FAME chromatographs here strongly indicate the presence of two different species residing in the pitcher plants.

The growth curves, antibiotic profiles, and FAME chromatographs consistently report two different species isolated from the pitcher plants. Furthermore, these isolates are similar but distinguishably different from *M. gordonae*, thus it was confirmed that two new likely novel species were discovered. These two novel species, represented by DL734 and DL739 are given the proposed names *Mycobacterium purpureae* and *Mycobacterium helvus,* respectively. After characterization of the novel species, the question of mycobacterial survival in the pitcher plant ecotope remained. Mycobacteria survival in fluctuating temperature and pH conditions is the topic of Part III.

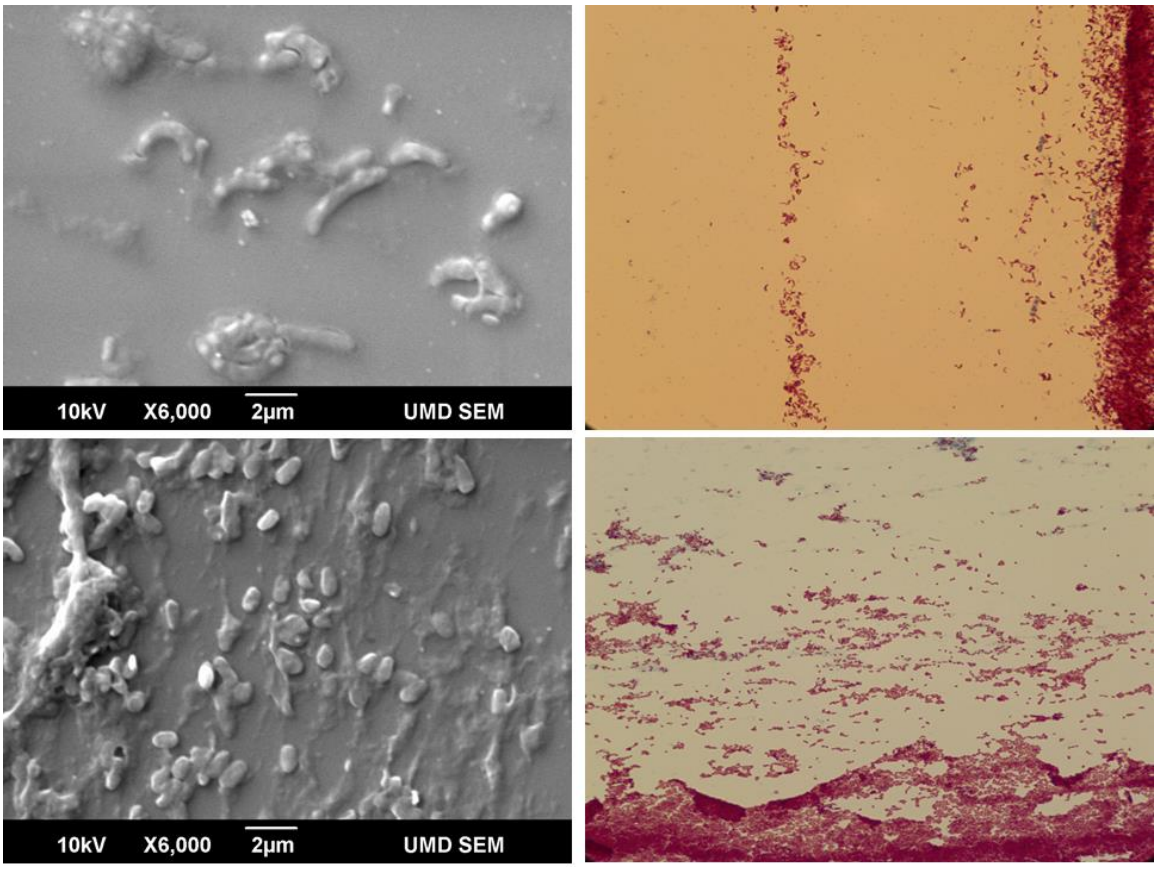

Figure 5. Scanning electron images and light microscopy images of DL734 (top) and DL739 (bottom). SEM images revealed DL734 cells were more pleomorphic in shape and size ranging from small bent-rod shaped cells to long rod cells (*top left panel*). DL739 exhibited uniform coccoidal to stout rod shaped cells smaller in size than DL734 (*bottom left panel*). Right panels are the acid-fast staining characteristics of DL734 (*top right panel)* and DL739 (*bottom right panel)*.

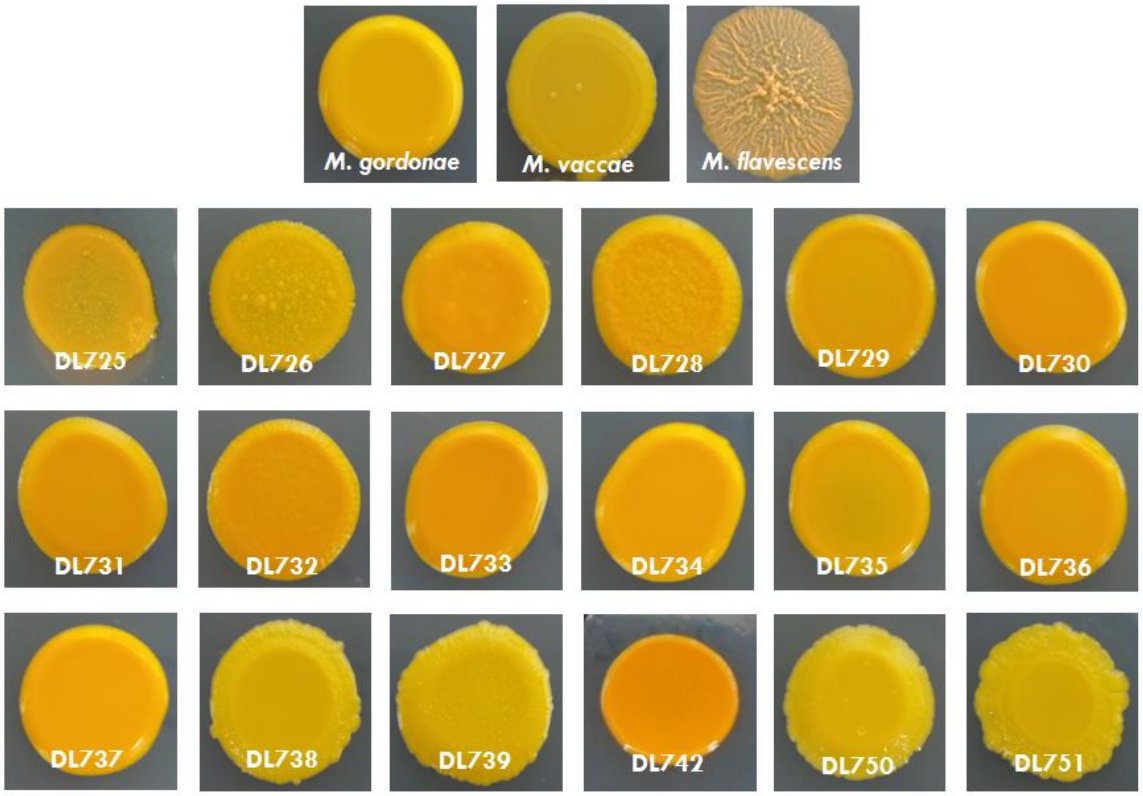

Figure 6. Colony morphologies of pitcher plant isolates, *M. gordonae, M. vaccae,* and *M. flavescens.* Colony spots were produced from 10-µL aliquots grown on 7H11 agar enriched with OADC. All images were taken 7 days after incubation at optimal growth temperatures with a Samsung Galaxy Camera 2 EK-GC200. Isolates DL725-DL737 were found in *S. purpurea* pitchers at Repose Lake and isolates DL738-DL751 were found in *S. purpurea* pitchers at the Laurentian Educational Center.

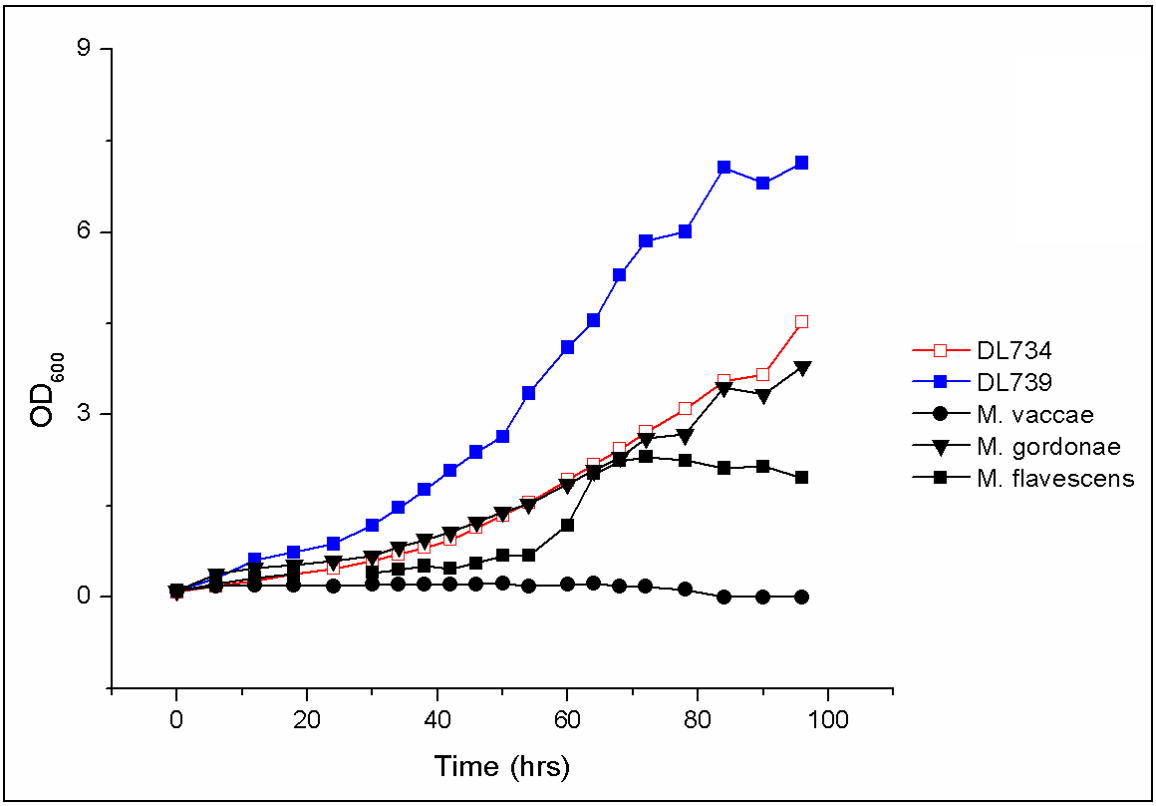

Figure 7. 28°C growth curves for DL734, DL739, *M. gordonae, M. flavescens* and *M. vaccae*. Growth curves were conducted over a period of five days and OD_{600} readings were recorded every 4-6 hours for 96 hours. Growth patterns exhibited by DL734 were not significantly different from *M. gordonae* (t = 0.81, P = 0.42) or *M. flavescens* (t = 1.44, P = 0.17), but slightly different from *M. vaccae* (t = 2.26, P = 0.04). In contrast DL739 was significantly different from *M. gordonae* (t = 3.12, P = 0.004), *M. flavescens* (t = 4.82, P = 9.08×10^{-5}) and *M. vaccae* (t = 5.27, P = 4.35×10^{-5}). The average growth rates for DL734 strains and DL739 strains were also distinctly separate (t = 2.56, P = 0.02).

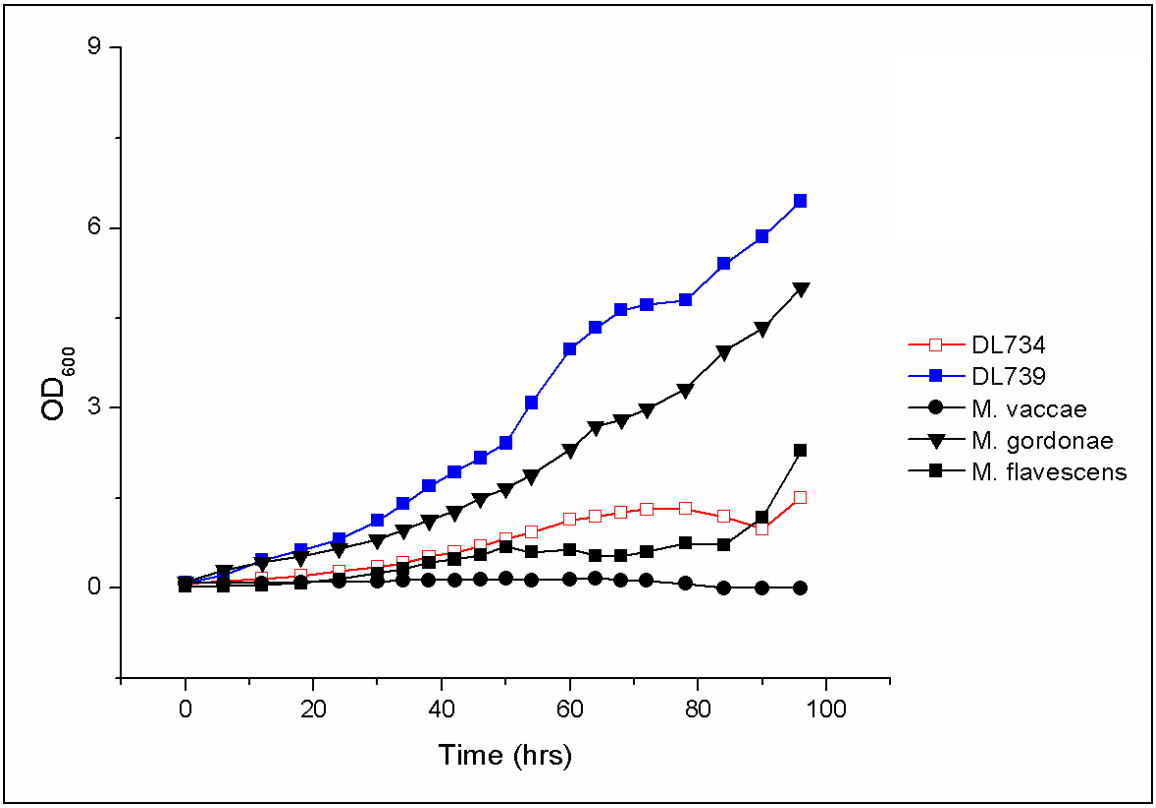

Figure 8. 32°C growth curves for DL734, DL739, *M. gordonae, M. flavescens* and *M. vaccae*. Growth curves were conducted over a period of five days and OD_{600} readings were recorded every 4-6 hours for 96 hours. Growth patterns exhibited by DL734 were not significantly different from *M. gordonae* (t = 1.89, P = 0.07). DL734 was slightly different from *M. flavescens* (t = 2.22, P = 0.04) and substantially varied from *M. vaccae* (t = 4.18, P = 3.8×10^{-4}). In contrast DL739 was significantly different from *M. gordonae* (t = 2.19, P = 0.03), *M. flavescens* (t = 2.35, P = 0.03) and *M. vaccae* (t = 5.15, P = 1.2×10^{-4}). The mean growth rates for DL734 strains and DL739 strains were also distinctly separate (t = 4.38, P = 2.6×10^{-4}).

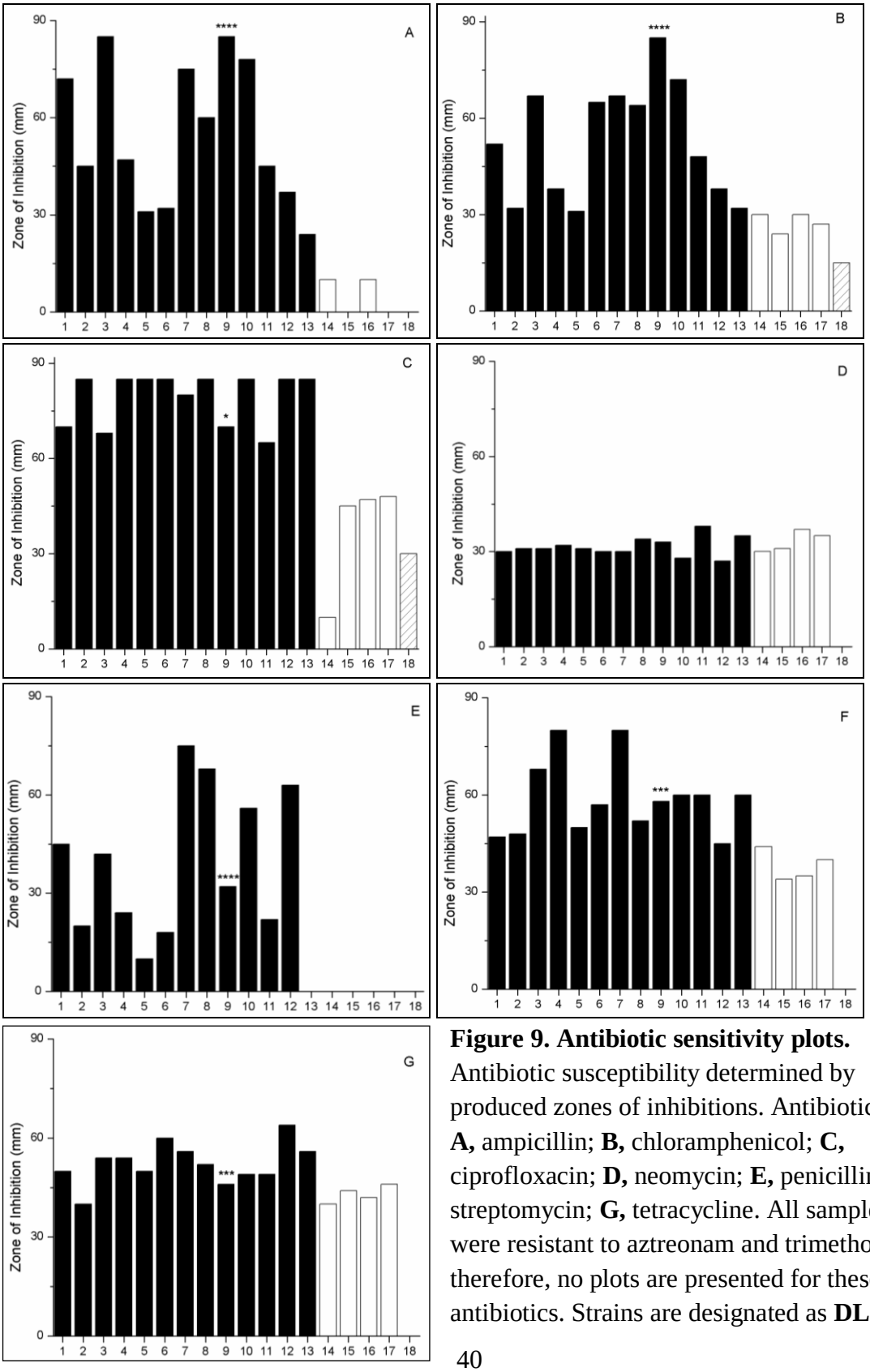

Figure 9. Antibiotic sensitivity plots. Antibiotic susceptibility determined by produced zones of inhibitions. Antibiotics are: **A,** ampicillin; **B,** chloramphenicol; **C,** ciprofloxacin; **D,** neomycin; **E,** penicillin; **F,** streptomycin; **G,** tetracycline. All samples were resistant to aztreonam and trimethoprim, therefore, no plots are presented for these antibiotics. Strains are designated as **DL734**

clade (filled bars): 1: DL725, 2: DL726, 3: DL728, 4: DL729, 5: DL730, 6: DL731, 7: DL732, 8: DL733, 9: DL734, 10: DL735, 11: DL736, 12: DL737, 13: DL742, **DL739 clade (empty bars):** 14: DL738, 15: DL739, 16: DL750, 17: DL751, and 18: *M. gordonae* (striped bar). Statistical significance categorized by $P \leq 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***), $P \leq 0.0001$ (****).

Table 3. Comparison of metabolic and biochemical profiles between pitcher plant isolates, *M. gordonae, M. vaccae,* and *M. flavescens*.

+: positive result, (+): weak positive result; +*: reaction occurred after 7 days, - : negative result

P: photochromogenic; S: scrotochromogenic; ND: no data

				DL734 Isolates														**DL739 Isolates**			
	M. gordonae	*M. vaccae*	*M. flavescens*	DL725	DL726	DL727	DL728	DL729	DL730	DL731	DL732	DL733	DL734	DL735	DL736	DL737	DL742	DL738	DL739	DL750	DL751
Growth at 28 C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth at 32 C	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+
Growth at 37 C	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+
Pigmentation	P	P	S	P	S	S	S	S	S	S	P	S	S	S	S	S	S	P	P	P	P
5% NaCl tolerance	-	-	+	-	-	-	-	-	(+)	(+)	(+)	-	-	-	-	-	-	(+)	(+)	(+)	(+)
Tween 80 hydrolysis	-	-	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+	+	+	+
Urease	-	+*	+	-	-	-	-	-	+*	-	-	-	-	-	+*	-	-	+*	+*	+*	+*
Nitrate reduction	+	+	+	(+)	-	-	-	+	+	+	+	+	-	(+)	+	-	-	+	-	-	-
3-day arylsulfatase	+	+	-	+	-	-	+	-	-	+	-	-	+	-	-	-	-	+	+	+	+
14-day arylsulfatase	+	+	ND	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+
Heat-stable catalase	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Tellurite reduction	-	-	ND	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 4. Comparative analysis of pitcher plant isolates with *M. gordonae* using FAME. Fatty acid content is reported as a percentage of total lipid composition.

Peak Identification	*M. gordonae*	DL729	DL730	DL731	DL734	DL736	DL737	DL738	DL739	DL742	DL750	DL751
10:0	-	-	1.31	-	0.94	1.63	0.94	1.23	0.88	1.07	1.18	1.01
12:0	3.75	-	0.94	-	0.73	0.97	0.92	2.73	1.49	-	2.54	2.36
14:0	4.34	5.95	8.20	8.17	8.04	7.41	7.47	8.00	6.39	7.75	8.32	7.43
2-Me-14:0	5.37	-	-	-	-	-	-	-	-	-	-	-
15:0	1.11	-	-	-	-	-	-	-	-	-	0.23	-
16:0	35.28	21.19	25.06	24.14	23.89	18.53	24.36	23.37	23.62	24.50	24.85	24.50
16:1 w6c	7.45	3.89	8.37	4.77	9.34	6.06	7.28	6.64	6.44	9.77	6.18	6.19
16:1 w7c	5.36	1.89	2.46	-	1.77	1.54	1.75	8.06	3.77	3.00	6.63	5.47
16:1 w9c	-	1.28	0.88	-	-	-	-	-	0.66	-	0.56	0.61
8-Me-16:0	-	-	-	-	-	-	-	4.69	2.44	-	1.88	2.11
17:0	1.93	-	-	-	-	-	-	-	-	-	-	-
17:1 w7c	-	11.87	16.98	24.24	20.81	31.53	21.51	15.55	15.57	16.77	15.50	15.11
17:1 w8c	0.56	-	-	-	-	-	-	-	-	-	-	-
18:0	2.66	1.39	1.68	3.20	2.36	1.75	2.10	2.38	1.82	3.35	3.17	2.92
18:1 w7c	-	1.50	-	-	-	-	-	-	-	-	-	-
18:1 w9c	30.50	39.52	25.08	22.80	20.61	15.39	21.80	12.51	14.24	19.39	15.06	14.97
18:2 w6,9c	1.69	5.47	1.06	-	1.04	-	1.00	-	0.50	-	0.89	0.58
10Me-18:0 TBSA	-	6.05	7.31	12.68	10.47	11.54	9.18	7.89	13.93	11.94	6.25	9.90
20:0	-	-	-	-	-	-	-	0.93	1.03	1.19	0.85	1.08
20:0 ALC 18.838 ECL	-	-	0.67	-	-	3.64	1.70	6.03	7.20	1.27	5.91	5.73

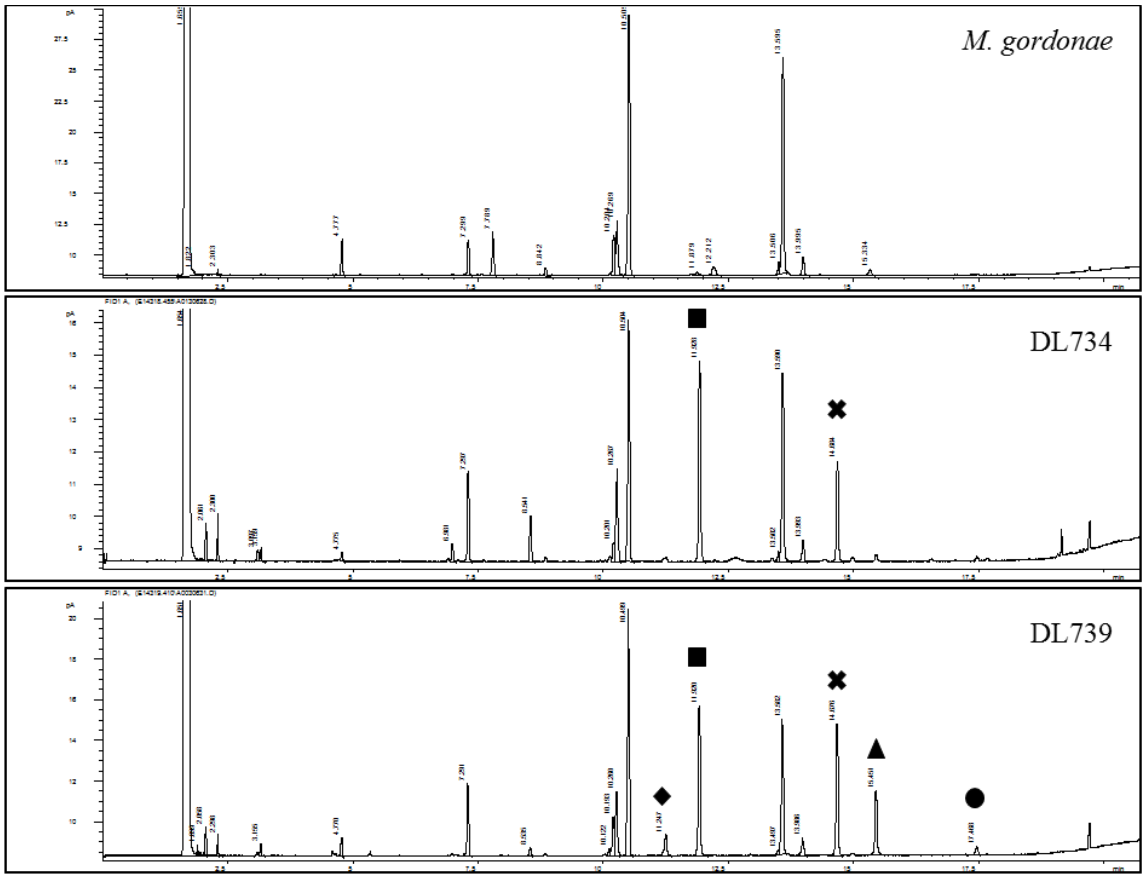

Figure 10. Characteristic FAME chromatographs for *M. gordonae* and pitcher plant type strains. FAME analysis was conducted at Microbial ID, Inc. (Newark, DE) by saponification, methylation, and extraction. Profiles were separated by gas chromatography, and identified using the Sherlock Microbial Identification System (MIDI, Inc.). The majority of the peaks are late-emerging forming single clusters. *M. gordonae* is the only profile to exhibit production of 2-Me-$C_{14:0}$, $C_{17:0}$, and $C_{17:1}$*w8c*. Inserted shapes indicate lipid species unique to pitcher plant isolates not found in *M. gordonae*. Peaks produced by all pitcher plant isolates are (■): $C_{17:1}$ *w7c*, (**X**): 10-Me-$C_{18:0}$. Peaks produced by only DL739 and associated strains are (♦): 8-Me-$C_{16:0}$, (●): $C_{20:0}$, and (▲): $C_{20:0}$ ALC 18.838 ECL, which was also produced by DL730 and DL736. Please refer to figures A4-A15 in the appendix for the complete set of FAME chromatographs.

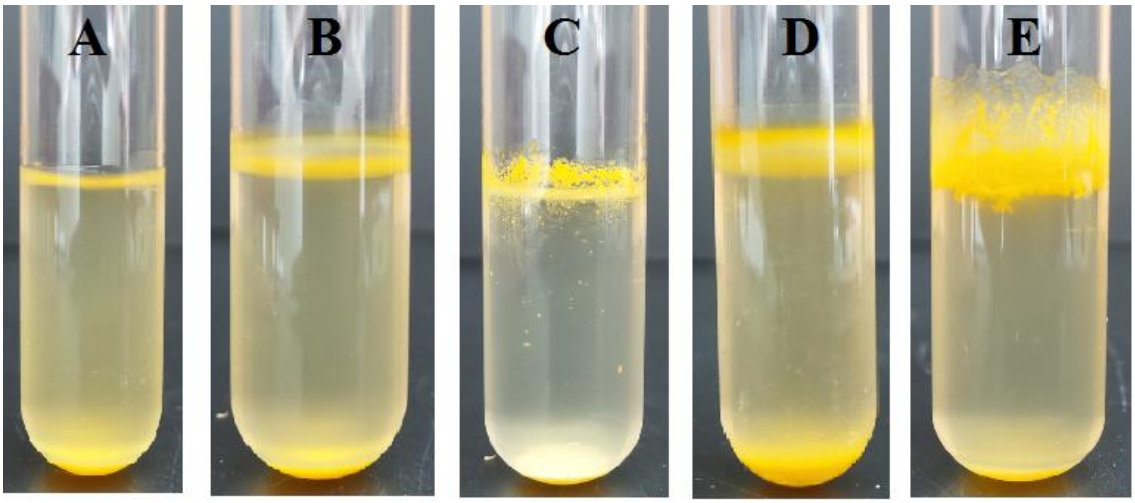

Figure 11. Representative biofilm production. All cultures were incubated at room temperature for two months without shaking in 7H9. A-E: cultures settled to the bottom and suspended throughout the media. **A**, representative of non-biofilm forming strains: DL728-DL730, DL732-DL735, DL738-DL750; **B,** representative of slight biofilm forming strains: DL726 and DL736; **C,** DL751 produces an irregular biofilm; **D,** biofilm former strains: DL725 and DL727; **E,** biofilm with vertical spreading strain DL731.

Chapter 4: Responding to Stressors Found in the Pitcher Plant Environment

4.1 Introduction

A major component of this study was to determine if mycobacteria could survive in the pitcher plant environment and if sporulation is mechanism for survival. The pitcher plants were chosen for two reasons. First, *S. pupurea* are regular inhabitants of Minnesota sphagnum bog ecosystems and mycobacteria are commonly found in sphagnum bogs globally. Since it is known that the pitcher plants harbor a diverse aquatic ecotope, the question was to determine if mycobacteria were a part of this community [39,42,44]. Secondly, *S. purpurea's* pitcher water is spatially defined, and contain several unique properties. Since *S. purpurea* do not excrete their own digestive enzymes they rely on the microbial and invertebrate community to break down arthropod prey implying that the water is relatively rich in available nutrients for microbes [68]. The pitcher waters are also highly acidic, thereby selecting for microorganisms tolerant to acidity. No mycobacteria have been discovered or assumed to inhabit highly acidic environments because they have been traditionally thought to prefer neutral pH despite their high density in sphagnum bogs. Therefore it was of great interest to determine if mycobacteria do survive in these acidic environments.

Duluth, LEC and SPSP samples were collected from July-September 2013 where the average air temperature was 65°C with a range of 29°C-89°C. Repose Lake samples were collected in August 2012 where the average air temperature was 62°C with a 37°C-89°C range. If the isolates are permanent inhabits of these regions then they would be expected to survive or tolerate some subset of this wide temperature range. While the air temperature may vary greatly, the bog water temperatures tend to stay more constant around 30°C. This is because bogs are water-logged environments and water's high specific heat capacity allows it to retain heat longer, therefore the temperature remains more constant than the fluctuating air temperature. The question of Chapter 4 was to

determine how mycobacterial species found in these plants survived this low pH and low temperature environment.

Two specific abiotic growth factors were examined in this present study. Temperature and environmental pH play crucial roles in bacterial metabolism directly affecting survival [69–72]. These two abiotic factors ultimately determine the range of environments that a bacterium can or cannot occupy. Despite being a relatively homogenous genus genetically, mycobacteria have been found to inhabit an excessively wide range of environments [1,13,26,38,73]. Moreover, findings that distinct mycobacteria species are found in only certain habitats suggest that mycobacteria are highly adapted to inhabit specific environmental niches. For instance, *M. avium* is an opportunistic mycobacteria commonly found in drinking waters. When these were grown in human macrophages, their virulence increases when compared to cells grown *in vitro* [5]. *M. avium* grown in a homogenous biofilm is more resistant to antibiotics than cells in solution [5]. Numerous independent studies of mycobacteria in human water systems tend to result in identification of the same species while different sets of mycobacteria are more commonly isolated from soils [12,18–20,74]. All environments can differ in bacterial assemblages, carbon sources, nutrient sources, and oxygen levels. Interestingly, the two variables that maintain relatively constant for bacteria are pH and temperature requirements.

It has been reported that most mycobacteria species grow best in the lab in slightly acidic pH (pH 5-6.5) [13,75]. It is speculated this could be due to the electronegativity of the outer cell surface charge at acidic pH versus the neutral cell surface charge at neutral pHs. If the cell is negatively charged then it will attract vital positively charged ions [13]. However no studies have been found indicating the genus can survive below pH 5. Additionally, most mycobacteria survive in a temperature range between 30°C–45°C. Early studies suggest *M. scrofulaceum, M. avium,* and *M. intracellulare* can grow at a reduced rate at 10°C but it was not determined if these cells remained viable [76]. The only officially described psychrotolerant mycobacteria is *Mycobacterium psychrotolerans. M. psychrotolerans* was isolated from a pond in Spain and can grow at 4°C [73].The fact that

M. psychrotolerans was only recently discovered indicates that there could be more cold tolerant mycobacteria species. To begin exploring survival mechanisms, mycobacteria sporulation was tested.

Sporulation is the long-term formation of endospores for survival in response to significant environmental stress or high cell density [77]. Endospore formation is an important and complex survival mechanism observed in many Gram-positive pathogens such as *Bacillus, Clostridium,* and *Orenia.* After multiple environmental signals are received, such as low extracellular oxygen or nutrient concentrations, changes in surrounding pH or oxidative species, and temperature changes, signaling pathways inhibit regular protein synthesis and initiate genomic DNA replication [78]. A thick inner membrane then forms around the replicated DNA and necessary resources where it becomes a forespore. Eventually the vegetative body dies and decomposes leaving the thick membrane capsule, called a spore. During the spore state growth and division are arrested, but in turn the spores are resilient to ionizing radiation, chemical solvents, and high temperatures [78,79]. Thus endospore formation is due to an intricate relationship between the environment and the microbes.

By textbook definition mycobacteria are non-sporulators. The idea of mycobacteria sporulation is enticing to researchers because of the bacilli's high resistance to chemical and biological stressors. Early long-term studies with mycobacteria indicated growth arrest and smaller colony formation suggesting sporulation could be part of the mycobacteria lifecycle. Only a couple of recent studies have indicated spore-like formations in starved mycobacteria [10,80]. In the studies, *M. marinum* [80] and *M. avium* subsp. *paratuberculosis* [10] were variously challenged with starvation, desiccation, and anoxia. *M. marinum* is an environmental species causing tuberculosis in ecotherms whereas *M. avium* subsp. *paratuberculosis* has become an obligate pathogen causing Johne's disease in cattle. Ghosh *et al*. (2009) and Lamont *et al.* (2012) described spore-like morphotypes through transmission and scanning electron microscopy and from increased expression of dipicolinic acid (DPA). DPA is a chemical compound that makes

up 5 to 15% of the dry weight of Gram-positive spores lending to wet heat, dry heat, and low pH resistance [10,80,81]. DPA production can easily be analyzed through colorimetric assays [82]. For sporulation to occur environmental signals need to interact with or penetrate the unique mycolic acid membrane.

In this study it was found that *S. purpurea* pitcher waters were highly acidic (see Chapter 2). Ranging from 2 to 4, the waters were as acidic as gastric juice yet contained a seemingly robust bacteria and invertebrate community. Pitcher water temperatures averaged 28°C. It became necessary to determine the isolates' temperature growth range to a higher resolution in addition to survival in acidic environments. The results indicated that DL734 and DL739 are highly sensitive to temperatures above 37°C. DL734 did not survive long term exposure at 8°C, but DL739 and associated strains remained viable. DL734 and DL739 cells survived short term exposure to a pH 2 environment but did not survive long term incubation in acidic environments. The isolates were sensitive to alkaline conditions. These results suggest DL734 and DL739 may be transient inhabitants of the pitcher plant or the pitcher water community provides alternative modes of survival, such as nutrients or proton neutralizing components that was not studied here.

4.2 Materials and Methods

4.2.1 Materials and Statistical Analysis

All Middlebrook 7H9 media in this chapter was supplemented with 10% albumin dextrose catalase (ADC) (v/v), 0.05% Tween 80 (v/v) and 0.2% glycerol (v/v). All Middlebrook 7H11 media was supplemented with 10% oleic albumin dextrose catalase (OADC) (v/v) and 0.2% glycerol. These will be referred to as 7H9 and 7H11, respectively.

All statistical significance tests compared means for DL734 strains and DL739 strains using the Students two-tailed *t* test assuming unequal variances and a *P* value of <0.05 was considered to show a significant difference.

Effects of Environmental pH on Growth

4.2.2 pH Shock

Deionized water was adjusted to the desired pH with 12 M HCl for acidic pH and pellet NaOH for basic conditions. One-mL aliquots of the solution were added to microcentrifuge tubes. Actively growing colonies were swabbed directly into the solution and incubated at room temperature. 100 µL of the solution was plated onto 7H11 plates every 15 min for 30 min. Time 0 was plated right after inoculation. Plates were incubated at 28°C or 32°C depending on growth requirements and analyzed for growth at 3, 5, and 7 days.

4.2.3 pH Growth

Seven mL of 7H9 broth was adjusted to the desired pH as described above in glass culture tubes. The media was inoculated with actively growing colonies and an initial OD_{600} reading was taken. The cultures were incubated at optimal growth temperatures. After 7 days, a final OD_{600} reading was taken and cultures were diluted and plated onto 7H11 to determine colony-forming units (CFUs). SEM images were taken for DL734 and DL739 at times 0, 30 min and 7 days following the protocol in Chapter 3.

Effects of Temperature on Growth

4.2.4 Heat Shock

Actively growing colonies were swabbed into 15mL conical tubes with deionized water and Tween 80. The solutions were hand-vortexed and allowed to settle. One-mL aliquots

of the monosuspended layer were distributed into microcentrifuge tubes, each for a specific time measurement to diminish contamination. Samples were incubated at temperatures ranging from 52°C – 62.5°C. Every 10 min for 1 hour, 100 µL of the samples were plated onto 7H11 and incubated for 5-7 days at 28°C. Time 0 was plated straight from the conical tube.

4.2.5 Tolerant Temperature Range

To determine the temperature range tolerated by mycobacteria, cells were grown at different temperatures ranging from 8°C to 52°C. Temperatures tested were 8°C, 28°C, 32°C, 37°C, 42°C and 52°C. The isolates were grown on 7H11 plates and incubated at the specified temperatures for 7 days. Additionally, 7H9 broth in glass tubes was inoculated with actively growing colonies. The cultures were incubated with daily shaking for 7 days. Initial and final OD_{600} readings were taken for 52°C as this was the highest temperature tested. Again to determine cell viability, cultures were plated onto 7H11 and incubated for 5-7 days at 28°C.

4.2.6 Cold Tolerance

As the pitcher plant isolates were discovered in northern Minnesota where winter is a regular occurrence, it was important to determine if the isolates could survive cold temperatures. Five-mL aliquots of 7H9 in glass tubes were inoculated with cells from colonies grown on 7H11 plates. An initial OD_{600} reading was taken, then the cultures were incubated at 8°C with daily shaking for 2 weeks. After two weeks the cultures were immediately transferred to incubate at 28°C with shaking for 7 days. OD_{600} readings were taken before and after the 28°C incubation. All samples were diluted and plated to determine surviving CFU/mL.

4.2.7 Dipicolinic Acid Colorimetric Assay

Dipicolinic acid (DPA) is a small molecule that makes up ~10% of Gram-positive endospores and is commonly used as an indicator of sporulation [81]. The presence of DPA was analyzed as previously described with modifications [82]. All pitcher plant isolates were grown on 7H11 for 26 days and on Arret and Kirshbaum (AK) #2 agar for 14 days at 28°C. *E. coli, B. megaterium,* and *B. subtilis* were inoculated onto AK agar and incubated at 37°C for 72 hrs. 100 mg (wet pellet weight) of each culture was suspended in 5 mL dH_2O then acidified with 0.1 mL of 4N HCl. The suspension was autoclaved for 20 min at 121°C and allowed to cool, then centrifuged at 3,600 *g* for 15 min. 4 mL of the supernatant was added to 1 mL of freshly prepared indicator buffer (1% (w/v) of ammonium iron(II) sulfate and 1% (w/v) L-ascorbic acid in 0.5M sodium acetate, pH 5.5). Solutions were centrifuged at 14,000 *g* for 20 min. OD_{440} measurements were recorded using *E. coli* as the blank because it is a known nonsporulator.

4.3 Results

4.3.1 pH Shock and Growth

All isolates showed no change in growth after 30-minute shocks at pH 2 or 4. Because of the continued survival at pH 2, a growth curve was conducted at this pH. After 7 days, DL734 strains averaged an OD_{600} increase of 0.09 while DL739 strains increased by .244 (Fig. 12). Despite the marginal increase in optical density measurements, no viable cells were recovered after the pH-adjusted growth. However, SEM images indicated that the cells are still present and intact at 30 minutes and 7 days after acid shock (Fig. 13). Interestingly, the cells have lost their hydrophobicity indicated by the lack of clumping. DL734 clumping decreased while cell swelling occurred. DL739 showed swelling at 30 minutes, but a return to normal cell size at 7 days. Cell density decreased for both strains as time increased indicated by the number of cells in the SEM samples (Fig. 13). These data suggest the isolates can tolerate at least 30 minutes of exposure to acidic

environments, but cannot indefinitely survive in such conditions. However, it is possible that the cells that survived 7 days of acid shock can permanently survive this environment or have entered some type of dormant state. All isolates failed to survive a pH 12 shock within 30 minutes.

4.3.2 Heat Shock and Tolerance

All isolates failed to survive heat shocks from 52°C – 62.5°C within 10 min as indicated by lack of growth on plated media. Control plates were overgrown with cells. DL734 and its isolates survived growths at 28-32°C while DL739 and its isolates survived temperatures ranging from 28-37°C. No isolates survived at 42°C or 52°C. A representative figure of the isolates growths' at 52°C indicates a constant decrease in cell density as measured by spectrophotometry (Fig. 14). No viable cells were isolated from the growths. Isolates at 8°C showed more complex results. Incubation at 8°C showed no visible growth, therefore it was determined that the cells were not psychrotolerant. However, after removing the isolates and allowing them to recover at room temperature, active growth was observed as indicated by increased turbidity of the media. After plating these suspensions and incubating at 28°C, cells were determined to be uncontaminated and viable. Therefore the cold tolerance test was conducted.

4.3.3 Cold Tolerance

Isolates were inoculated into liquid media and incubated at 8°C for 2 weeks. The isolates were then immediately transferred to incubate at 28°C for 7 days with no recovery time at room temperature. After 8°C incubation, a lack of turbidity indicated a lack of active cell replication which is represented by the minimal change in OD_{600} absorbances (Fig. 15). Cell density in CFU/mL remained the same for DL739 and decreased by a magnitude for *M. gordonae, M. vaccae,* and *M. flavescens.* No DL734 strain produced colonies after this incubation period. Visually, turbidity increased after 28°C incubation (Fig. 15). Cell density however fell from $3.7x10^8$ to $5.9x10^5$ for DL739 strains and one magnitude for *M. gordonae, M. vaccae,* and *M. flavescens.* DL734 cell density, interestingly, increased by a

magnitude. The lack of colonies after 8°C followed by signs of cell recovery could indicate that DL734 entered viable but non-culturable state at the colder temperature.

4.3.3 DPA Assay

The presence of dipicolinic acid (DPA) in the endospore wall is characteristic of all Gram-positive sporulating bacteria. Therefore, it is a useful marker to detect spore formation. The colorimetric procedure utilizes a visual color change due to the binding of ammonium iron(II) sulfate with pyridine-2,6-dicarboxylic acid (DPA) under acidic conditions. Higher concentrations of DPA will exhibit a more drastic color change which can be detected by a spectrophotometer. All pitcher plant isolates formed colonies on 7H11 and AK #2 sporulating agar plates. *B. subtilis* and *B. megaterium* produced detectable levels of DPA after 72 hours on AK #2 agar (OD_{440} of 0.113 and 0.017, respectively) (Fig. 16). The pitcher plant isolates produced zero to low levels of DPA when grown on 7H11 and only slight amounts of DPA when grown on AK #2 agar (Fig. 16). Interestingly, DL725 and DL726 grown on AK #2 agar produced DPA levels similar to *B. megaterium* with OD_{440} readings of 0.01 and 0.013, respectively (Fig. 16). As this was preliminary results, replications were not done and statistical significance could not be inferred.

4.4 Discussion

Determining the ability of mycobacteria to survive extreme pHs and temperatures is relevant in terms of mycobacteria pathogenicity. This is an important debate to resolve as the physical state of *M. tuberculosis* and its apparently many survival mechanisms within the host macrophage has not been completely elucidated. There are several theories that attempt to decipher exactly how *M. tuberculosis* persists in aveolar macrophages, but it is reasonably clear that the pathophysiology of tuberculosis includes the prevention of phagosome-lysosome fusion into an acidic phagolysosome [83,83,84]. Phagolysosomes are vesicles filled with active nitrogen and oxygen reactive species used to degrade biological

materials. All of the theories on mycobacteria survival mechanisms are based on the assumption that *M. tuberculosis* cannot survive the acidic stress that is caused by exposure to the lysosome. To support this assumption, it has been shown by multiple groups that *M. tuberculosis* survives in macrophages by preventing the pH decrease that acts as a signal for phagosome-lysosome fusion [30,32,85]. Additionally, *M. tuberculosis* exhibits multiple different transcriptional responses to pH fluctuations indicating that pH is a major environmental cue for survival and virulence. However, it is unclear if responses to pH affect cell metabolism and physiology. The current debate attempts to determine if the *M. tuberculosis* bacilli enters a dormant, non-replicative state, or a spore-like state similar to Gram-positive sporulation [10,80,86–89]. The early results of this chapter would suggest the bacilli enter a metabolically inactive, dormant state. The dormant state could be the mechanism used by the pitcher plant isolates to survive within the *S. purpurea* pitchers waters.

4.4.1 Effects of pH on Growth

My findings suggest DL734 and DL739 present similar phenotypes in response to fluctuations in pH. All isolates temporarily tolerated an acidic solution and failed to replicate in alkaline environments. This indicates that the isolates have developed a mechanism to counteract a large proton influx gradient that does not produce the same effect in a large proton efflux gradient. The isolates are unable to cope with proton loss to the environment. The decreased growth rate in acidic conditions strongly suggest these strains do not have the capacity to survive acid stress, however it could also indicate that some cells are entering a dormant or non-replicative state triggered by the excess protons from which recovery was not seen. It would be necessary to determine if loss in survival was due to the sudden exposures and if responses would be different to a gradual decrease in environmental pH.

In any given environment mycobacteria will attempt to maintain neutral intracellular pH [90,91]. Therefore, protons flooding the cell could alter its interior pH disrupting regular enzymatic functions of various proteins. Previous studies have indicated several genes,

including the *M. tuberculosis* Rv3083-to-Rv3089 operon, are upregulated in response to acid stress [92,93]. The Rv3083-to-Rv3089 operon is putatively involved in fatty acid metabolism and was induced within 15 minutes of an acid shock [92]. The relatively short timeframe for *M. tuberculosis* to induce preferential gene expressions in response to acid shock would suggest that these new strains, DL734 and DL739, do not harness these genes and cannot cope with acid stress in this same fashion. Furthermore, changes in the identities or induction of several proteins in response to pH fluctuations indicate mycobacteria have evolved to minimize pH damage to the cell [30,94,95]. Some of these proteins could be important for metabolism hence inhibiting growth [95,96]. However transcriptional regulation of DL734 and DL739 was not a part of this study and should be pursued in the future.

4.4.2 Effects of Temperature on Growth

Temperature responses for the pitcher plant isolates were more varied. No isolates tolerated growth above 37°C. However, DL734's temperature range is more restricted than DL739. DL734 can only grow between 28°C-32°C whereas DL739 can grow in temperatures ranging from 28°C-37°C. This is a fundamental difference in bacterial metabolism between these two isolates and is a strong indication that they are two distinct species. Despite this difference, both species show the same response to cold temperatures. Cells slightly replicated within the 14 days of cold incubation indicated by a minimal increase in optical density. Once removed from the cold, the cells showed immediate growth recovery indicating that while in the non-replicative state, the cells still continue to sense and respond to the surrounding environment. This would suggest that there is little damage to the cells caused by cold temperatures. However, cell densities of most strains decreased by at least a magnitude after the recovery incubation indicating some cell death. It was not determined if the cellular density was a result of the colder temperatures or starvation. Interestingly, DL734 increased in cell density after removal from 8°C indicating there was no excess cell death. Cells remained viable at all points of the growth.

4.4.3 Dormant State as a Mode for Survival

These results suggest that some mycobacteria, such as DL734, have the capacity to enter a dormant state of growth, and that this dormant state may be responsible for mycobacteria survival in low pH, low temperature environments–such as sphagnum bogs and pitcher plants. Recent research agrees on the possibility of a mycobacteria dormant state, but the physical nature of this state remains contentious. One theory is that mycobacteria are spore-formers forming highly resistant endospores similar to *Bacillus* and *Clostridium* species [10,80]. These studies showed changes in cell morphology, production of dipicolinic acid (DPA), and the lack of cell division. However, other research groups have failed at reproducing the results from these studies weakening the theory [97]. Here, mycobacteria pitcher plant strains produced inconclusive results. First, the malachite green strain for endospores varied between strains (Fig. A16). All strains in the DL734 clade produced positive malachite green stains indicated by the presence of green staining cells. DL750 produced positive malachite green cells whereas DL751 produced negatively stained cells. However, cell morphology as seen by light microscopy appeared unchanged. Either the malachite green stain for sporulation does not work for mycolic acid containing cells, or pitcher plant isolates are producing structures that retain the malachite green dye. Therefore, to determine the structure of the cells, DPA production was examined. Dipicolinic acid is the most common lipid species in endospores of *Bacillus*, which is why it is often used as a sporulation indicator [81]. The lack of DPA production by the pitcher plant isolates does not lend support to the spore-forming hypothesis. No production of DPA by mycobacteria does not necessarily discredit the sporulation hypothesis because *Mycobacterium* and *Bacillus* are two different genera, but it does indicate methods used for other species may not be compatible for mycobacteria, and that mycobacteria sporulation may arise from a mechanism different from *Bacillus* species.

Another theory maintains that cells do not change morphology, but rather become metabolically inactive [98,99]. In fact a state of metabolic stasis is the basis for the Cornell model of *M. tuberculosis* latency [100]. *M. tuberculosis* may infect an individual

asymptomatically until they become immunosuppressed, whereby the bacilli begins to replicate and cause active tuberculosis disease. During the asymptomatic, latent phase of infection, it is believed the bacilli do not replicate. The pitcher plant isolates behaviors tend to indicate this model as a possibility for their survival. Response to acid shock would indicate the cells do not survive the exposure. SEM images of cells after acid shock do not indicate active cell replication (Fig. 13). Overall cell density decreased as indicated by the number of cells in the SEM sample, but there is some evidence of possible sporulation for DL734. After 30 minute acid shock the cells showed more heterogeneous morphologies with some forming circular or club-shaped cells. However, this does not last into 7 days as the cells appear to revert to a normal shape. There is not enough evidence at this time for this result to be conclusive. DL734 and DL739 appear to have lost their electronegativity, indicated by the lack of clumping, and at least DL734 has temporarily enlarged in size. SEM cannot distinguish living from dead cells, therefore the images in conjunction with viable colony counts indicate that cells are no longer viable at some time point between 30 minutes and 7 days of exposure. However, cells were able to recover after a two-week cold shock seemingly with little decrease in cell population. The curiosity here lies in the fact that there was no active cell replication while in the cold, yet cells quickly recovered once incubation was returned to optimal temperatures. Taken together, the pitcher plant isolates show varying responses dependent on the stressor, and remain metabolically inactive at least in response to temperature. While studies have provided glimpses to solve the mysterious state of the bacilli during infection, the question remains unanswered.

Though traditionally classified as mesophilic bacteria that prefer neutral pH environments, the pitcher plant isolates have demonstrated that the classic definition may be too restrictive. Perhaps phenotypically distinct species such as *M. psychrotolerans, M. minnesotense,* and *M. helvus* and *M. purpureae* presented here, are more common than previously thought. Even though sphagnum bogs are the single most common mycobacterium habitat, mycobacteria from the bogs are currently considered the exception and not the norm [1]. It would be beneficial to revisit many environmental and

opportunistic pathogenic mycobacteria and test their ability to withstand proton and cold shock in order to elucidate survival mechanism related to disease states.

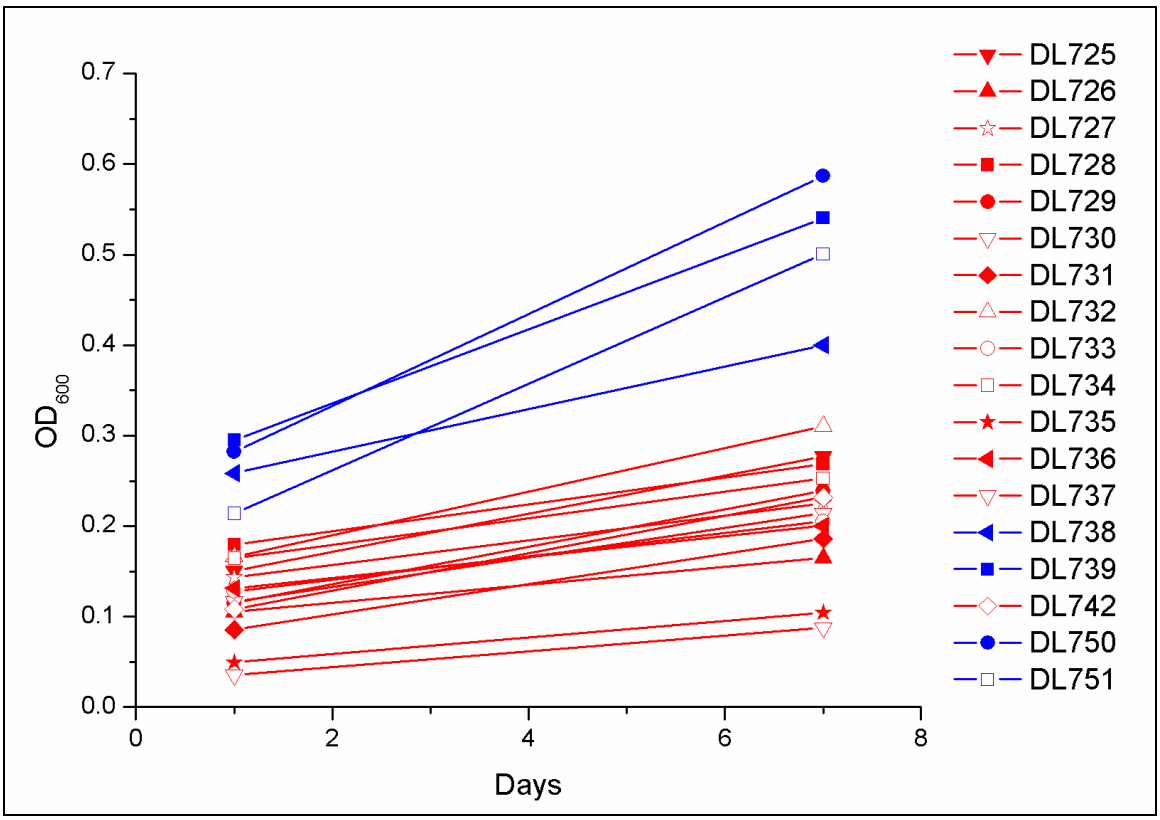

Figure 12. Growth at pH 2. Pitcher plant isolates were allowed to grow in 7H9 adjusted to pH 2 for 7 days. OD_{600} was recorded at the time of inoculation and after 7 days. Data points are the average of two replicates. The rise in absorbance did not correlate to cell survival. No cells survived the pH-adjusted growth. There was a significant difference in the acid shock response between the DL734 and DL739 strains P = 0.02, t = 4.08. Blue, DL739 isolates; red, DL734 isolates.

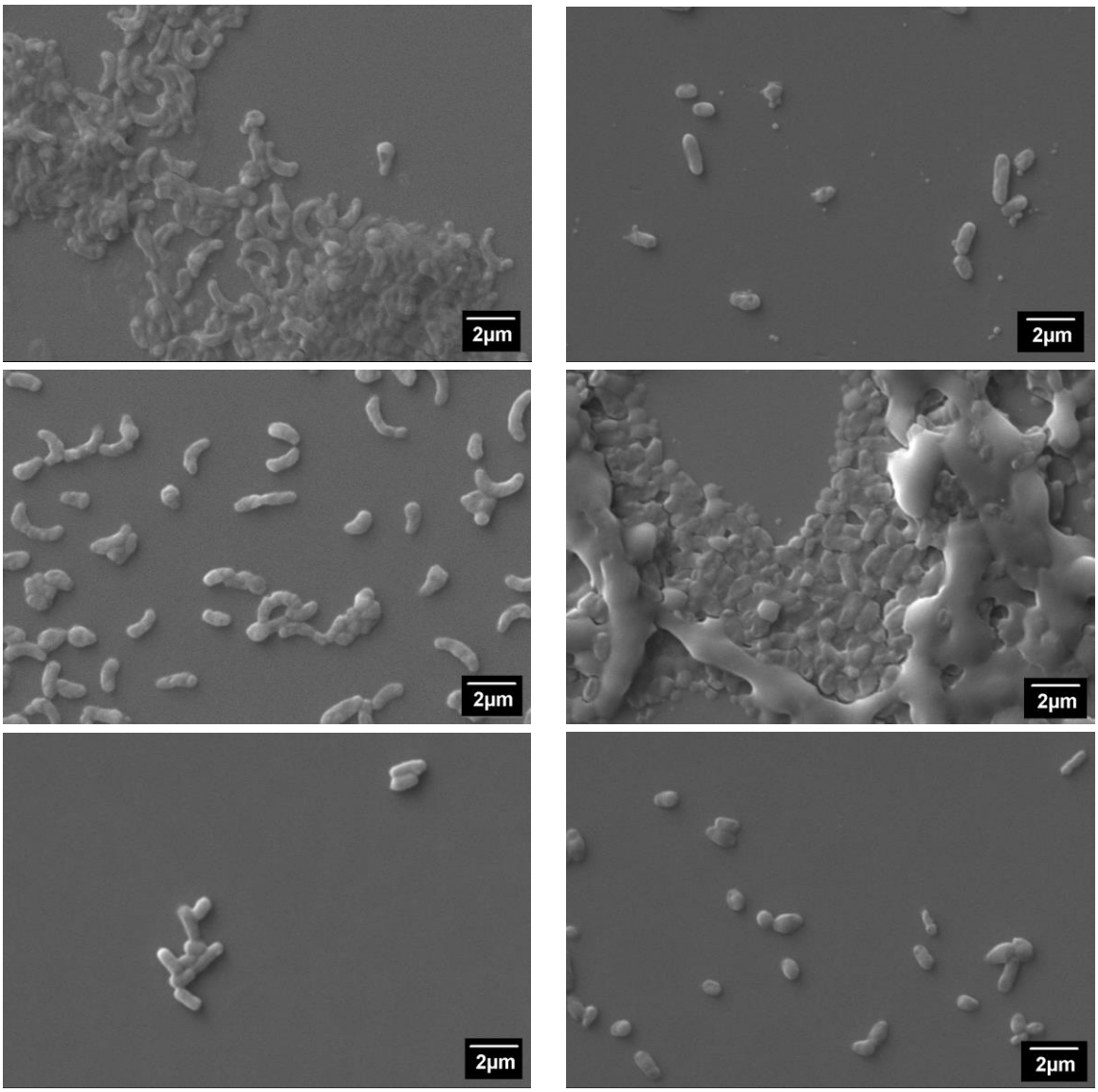

Figure 13. SEM images of DL734 (left) and DL739 (right) before and after pH shock. *Left Top:* DL734 before shock. Cells are clumped with homogenous morphology. *Left Middle:* DL734 after 30 min shock. Cell density and clumping decreased while size has increased. Cell morphology became heterogeneous with some shrinking into round or clubbed cells. *Left Bottom:* DL734 after 7 days shock. Cell numbers decreased with a general return to regular cell morphology. *Right Top:* DL739 before acid shock. Cells are clumped with homogenous morphology. Differences in cell size are likely due to division as small cells are half the length of the larger cells. Image is not representative of cell density, which more resembled DL734. *Right Middle:* DL739 after 30 min shock. No change in cell density, clumping, or cell size. There is a thick mat covering the cells from the fixation process. This is not a cellular product. *Right Bottom:* DL739 after 7 days shock. Cell density has decreased from 30 min with no change in cell morphology.

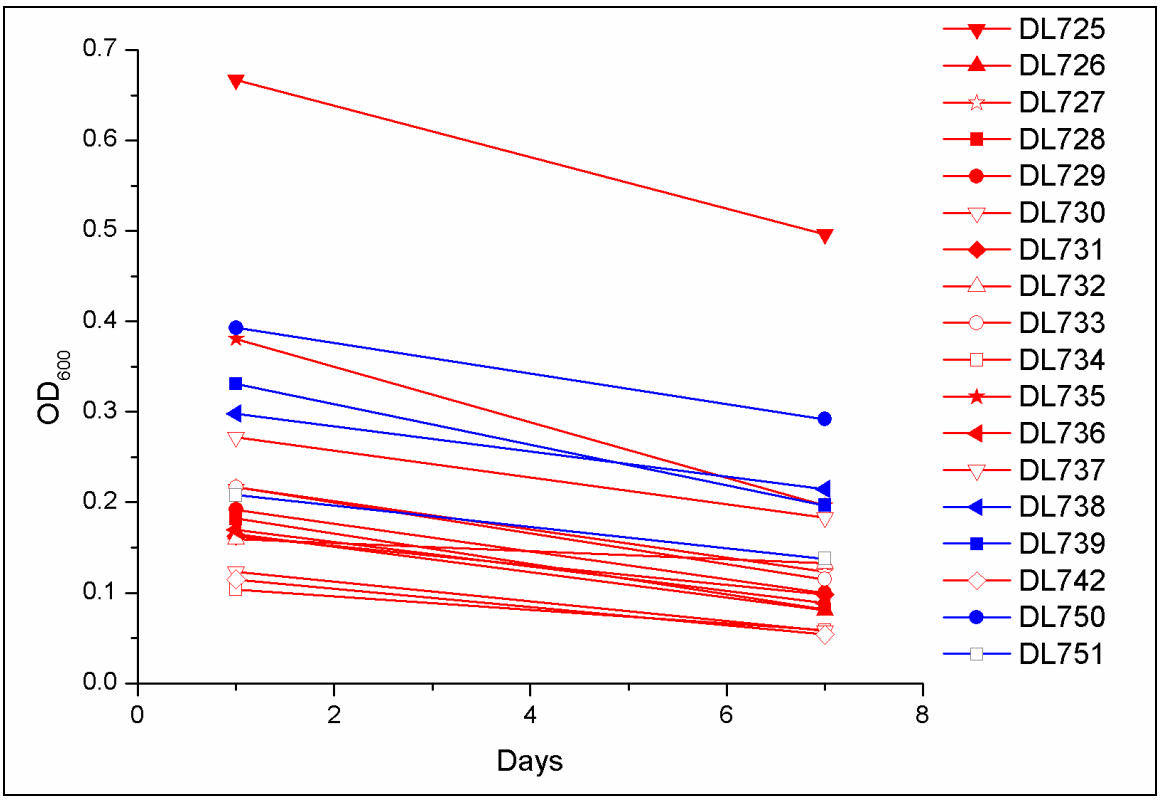

Figure 14. Growth at 52°C. Pitcher plant isolates were allowed to grow in 7H9 for 7 days with shaking at 52°C. OD_{600} readings were taken at time of inoculation and after 7 days. No isolates survived growth at this elevated temperature. Blue, DL739 isolates; red, DL734 isolates.

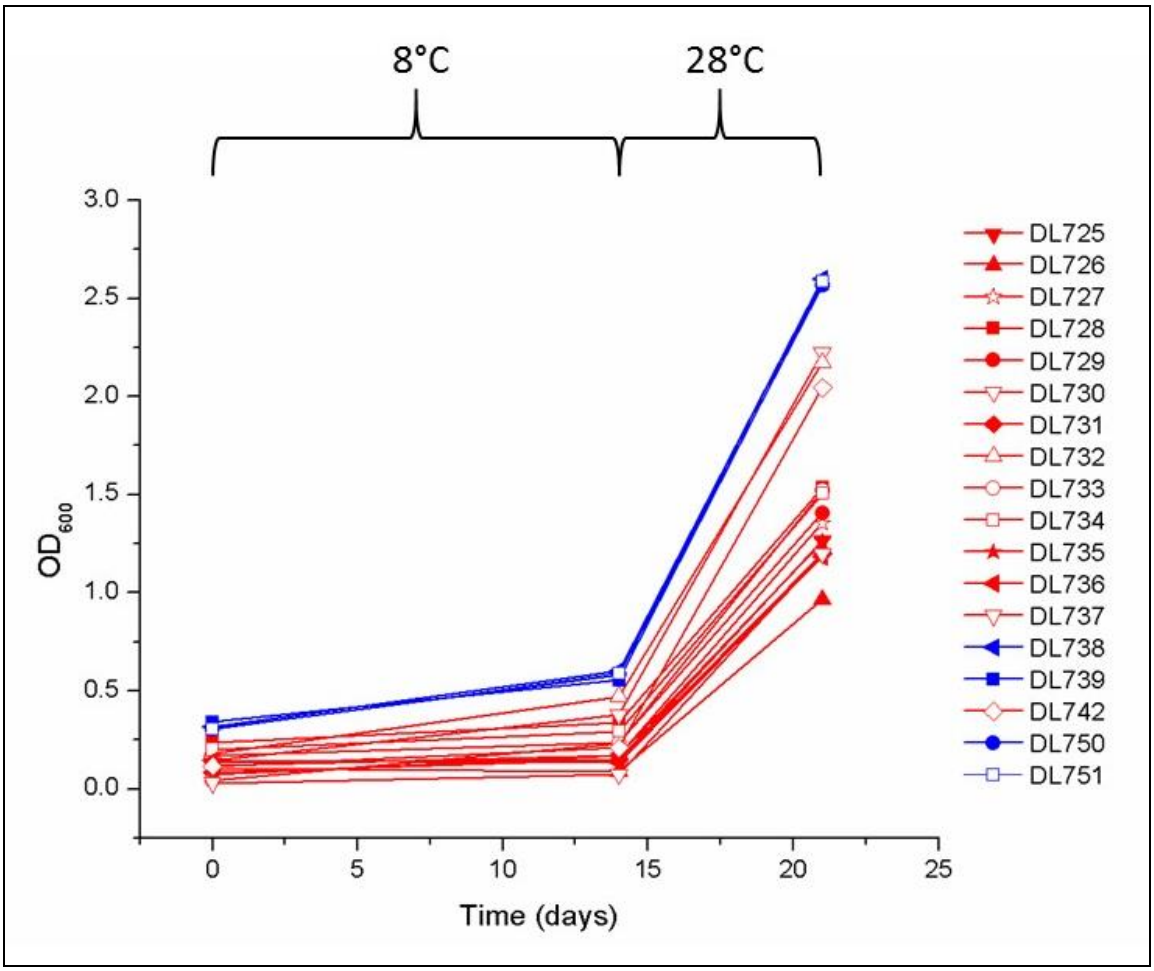

Figure 15. Cold tolerance demonstrated by pitcher plant isolates. Pitcher plant isolates were allowed to grow in 7H9 for 14 days with daily shaking at 8°C. Cultures were then moved to 28°C incubation for 7 days. OD_{600} readings were taken at time of inoculation, before and after 28°C incubation. Little cell replication was seen after 8°C, but absorbance increased after 28°C incubation. Blue, DL739 isolates; red, DL734 isolates.

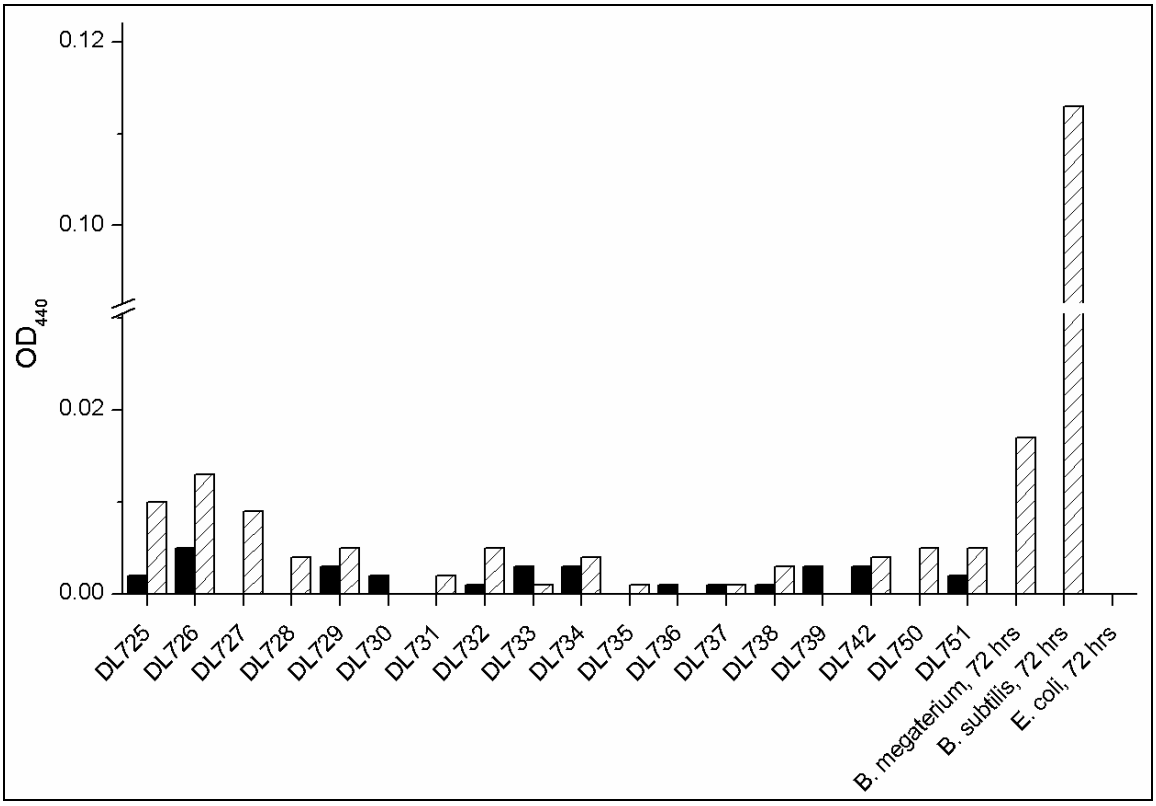

Figure 16. Dipicolinic colorimetric assay. OD_{440} readings were taken for all pitcher plant isolates, *B. subtilis*, *B. megaterium*, and *E. coli* grown on 7H11 (black bars) or AK #2 (dash bars) agar plates. *E. coli* was used as the blank and the *Bacillus* species were included as positive standards. High optical density correlates to high DPA density. OD_{440} for *B. subtilis* was 0.113 and 0.017 for *B. megaterium.* Mycobacteria isolates did not produce comparable levels of DPA except for DL725 and DL726 grown on AK #2 agar. DL725 had an OD_{440} of 0.01 and DL726 was measured at 0.013.

Conclusion

This is the first study focusing on mycobacteria in *Sarracenia purpurea* aquatic ecosystems. This study positively identified several isolates of mycobacteria from pitcher plants located in two different regions of Minnesota. The isolates are mainly isolated to a specific region and show limited species dispersal. This is in line with previous studies that have shown water ecosystems within the pitchers vary from pitcher to pitcher even within the same habitat [44].

Two novel mycobacteria species were discovered in *S. purpurea* pitcher waters isolated from Minnesota northern sphagnum bogs. The two strains, designated DL734 and DL739, proposed are *Mycobacterium purpureae* and *Mycobacterium helvus,* respectively. *M. purpureae* was chosen for the larger clade because of their origin from the pitcher plant *S. purpurea. M. helvus,* helvus being Latin for light yellow, was chosen for DL739 because this clade produced light yellow pigmented colonies. The two strains displayed distinctly unique fatty acid profiles, growth curves and biochemical properties. Separation into two species is reinforced by phylogenetic analyses where two unresolved sister clades emerged separate from other established *Mycobacterium* species.

M. helvus and *M. purpureae* exhibit properties that indicate the ability to enter a dormant state in response to cold and acidic conditions. Response to acid shock is temporary and cells were unable to survive after long term exposure. However, both strains were able to survive long exposure to low temperatures and then recover at more hospitable temperatures. This is the first report of such an ability in mycobacteria and could indicate that dormant metabolic states are the primary survival mechanism within the pitcher plants. These strains also do not produce dipicolinic acid indicating sporulation as an unlikely survival mechanism. The bacterial and invertebrate community in Minnesota pitcher plants will need to be better characterized, and the chemical biology of the waters analyzed, in order to determine if there are any biotic and abiotic factors promoting mycobacteria survival. Pitcher plant waters are highly acidic, therefore, either these isolates have devised a means to survive long term, or are transient inhabitants. If *M.*

purpureae, *M. helvus*, and all other bog-inhabiting strains are indeed surviving in this niche, then these strains could be a useful tool in studying mycobacteria defenses against acid and cold shock.

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Appendix Table of Contents

Appendix A. Supporting Figures for Chapter 2

Table A1. Type strains and accession numbers used in phylogenetic studies. All accession numbers are as provided on NCBI (http://www.ncbi.nlm.nih.gov/).

Name	**Type Strain**	***dnaJ***	***rpoB***	***hsp65***
Mycobacterium alvei	CIP 103464	AB292562.1	AY859697.1	AF547805.1
Mycobacterium africanum	ATCC 25420	AB292525.1	JF923622.1	FJ617583.1
Mycobacterium arupense	DSM 44942	AB292527.1	EU191924.1	AB239922.1
Mycobacterium asiaticum	DSM 44297	AB292526.1	AY544884.1	AF547806.1
Mycobacterium avium sub. *avium*	ATCC 25291	AB292528.1	EF521907.1	GQ153289.1
Mycobacterium branderi	ATCC 51789	AB292531.1	JQ599259.2	AJ310223.1
Mycobacterium celatum	ATCC 51131	AB292533.1	AF057458.1	JF491292.1
Mycobacterium chelonae	ATCC 14472	AB292565.1	EU591505.1	FJ607870.1
Mycobacterium chimaera	CIP 107892	AB292534.1	GQ153309.1	AY943198.1
Mycobacterium chitae	CIP 105383	AB292566.1	AY544899.1	AF547819.1
Mycobacterium cookie	CIP 105396	AB292535.1	AY544904.1	AF547824.1
Mycobacterium farcinogenes	DSM 43637	AB456563.1	AY544910.1	AY299150.1
Mycobacterium flavescens	CIP 104533	AB292567.1	AY544911.1	AF547831.1
Mycobacterium fortuitum sub. *Fortuitum*	CIP 104534	AB292568.1	AY544913.1	AY458072.1
Mycobacterium gastri	CIP 104530	AB292536.1	AY544916.1	AF547836.1
Mycobacterium genavense	ATCC 51233	AB292537.1	AF057467.1	AY299183.1
Mycobacterium gilvum	DSM 44503	AB292569.1	AY544917.1	AF547838.1
Mycobacterium gordonae	ATCC 14470	AB292538.1	JF346873.1	AF434734.1
Mycobacterium haemophilum	CIP 105049	AB292539.1	AY544920.1	AF547841.1
Mycobacterium hiberniae	ATCC 49874	AB292540.1	JN571246.1	JN571198.1
Mycobacterium intermedium	ATCC 51848	AB292542.1	JF712874.1	AY299187.1
Mycobacterium intracellulare	AFP-000224	AB292543.1	JX294410.1	JX294392.1
Mycobacterium kansasii	ATCC 12478	AB292544.1	EU591500.1	JF491300.1
Mycobacterium lentiflavum	ATCC 51985	AB292546.1	JN881350.1	AY373453.1
Mycobacterium malmoense	ATCC 29571	AB292548.1	GQ153314.1	GQ153293.1
Mycobacterium marinum	ATCC 927	AB292549.1	AB548716.1	AY299134.1
Mycobacterium microti	CIP 104256	AB292550.1	AY544944.1	AY299135.1
Mycobacterium minnesotense	DSM 45633	JN587527	NA	JN546612
Mycobacterium mucogenicum	ATCC 49650	AB292571.1	AY147170.1	AY299155.1
Mycobacterium nonchromogenicum	ATCC 19530	AB292551.1	JN881351.1	JN571193.1
Mycobacterium parafortuitum	CIP 106802	AB292573.1	AY544952.1	AF547864.1
Mycobacterium phlei	ATCC 11758	AB292575.1	AF057480.1	AY299158.1
Mycobacterium porcinum	ATCC 33776	AB292577.1	AY544955.1	AY496137.1
Mycobacterium rhodesiae	CIP 106806	CP003169.1	AY544958.1	AF547870.1
Mycobacterium scrofulaceum	ATCC 19981	AB292552.1	GQ153305.1	GQ153288.1
Mycobacterium septicum	DSM 44393	AB292579.1	AY147167.1	AY373457.1
Mycobacterium simiae	ATCC 25275	AB292554.1	GQ153313.1	GQ153292.1

Name	**Type Strain**	***dnaJ***	***rpoB***	***hsp65***
Mycobacterium smegmatis	ATCC 19420	AB292580.1	AY262735.1	JF491307.1
Mycobacterium szulgai	ATCC 29716	AB292555.1	JN881348.1	JN881348.1
Mycobacterium terrae	ATCC 15755	AB292556.1	EU591502.1	AY299142.1
*Mycobacterium tuberculosis**	H37Rv	AL123456.3	AL123456.3	AL123456.3
Mycobacterium ulcerans	ATCC 19423	AB292559.1	EU591495.1	AY299145.1
Mycobacterium vaccae	ATCC 15483	AB292581.1	JF923624.1	JF491312.1
*Corynebacterium diptheriae gravis**	NCTC 13129	BX248353.1	BX248353.1	BX248353.1

* Gene sequences were obtained from the complete genome

NA: Not applicable

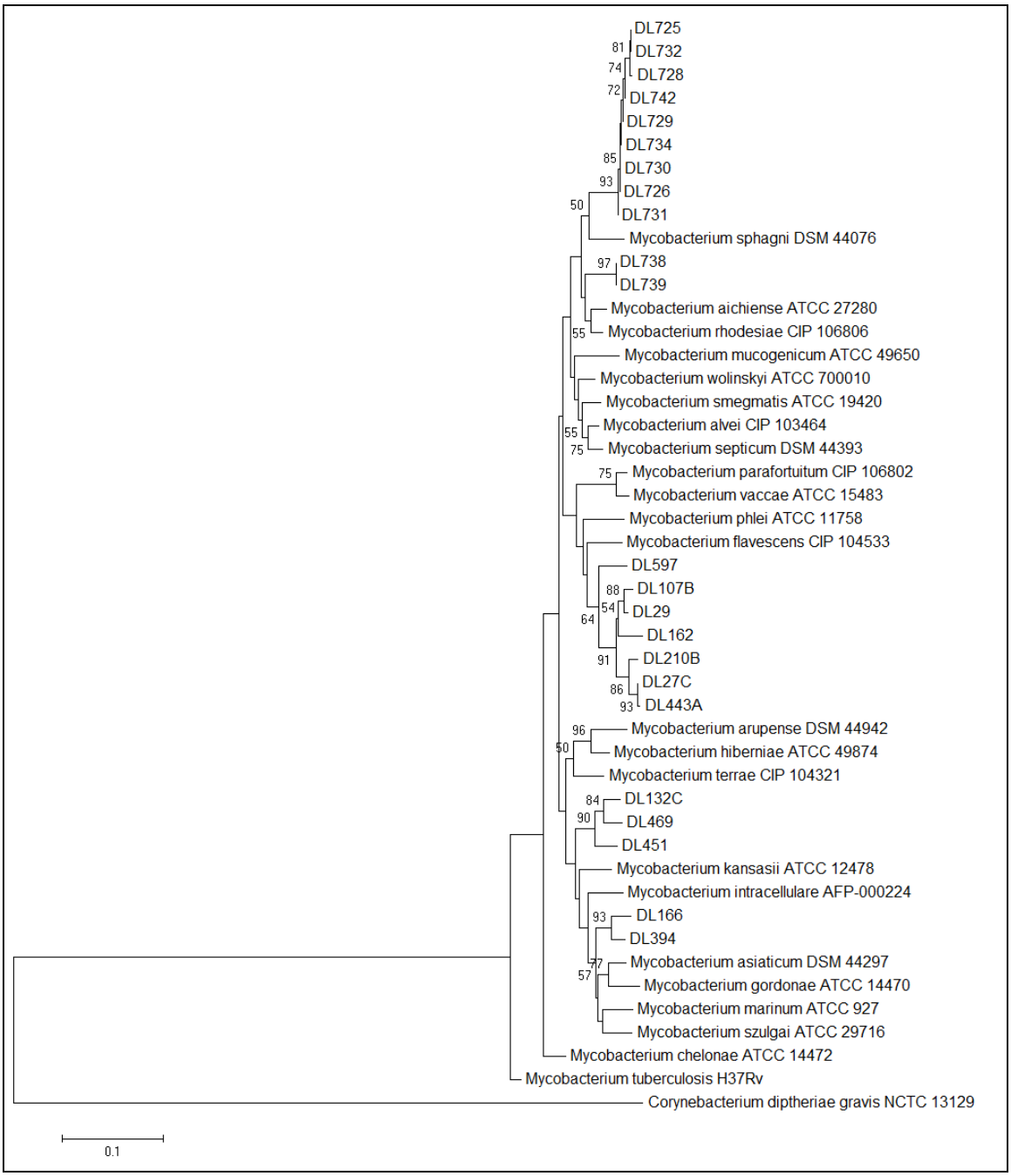

Figure A1. Phylogenetic analysis of pitcher plant isolates, bog isolates, and *Mycobacterium* species using the *hsp65* gene. The evolutionary history was inferred using the Neighbor-Joining method with *M. minnesotense* associated strains: DL27C, DL29, DL107B, DL132C, DL162, DL166, DL210B, DL394, DL443A, DL451, DL469, and DL597. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The evolutionary distances were computed using the Kimura 2-parameter method. Evolutionary analyses were conducted in MEGA6 [49].

Appendix B. Supporting Figures for Chapter 3

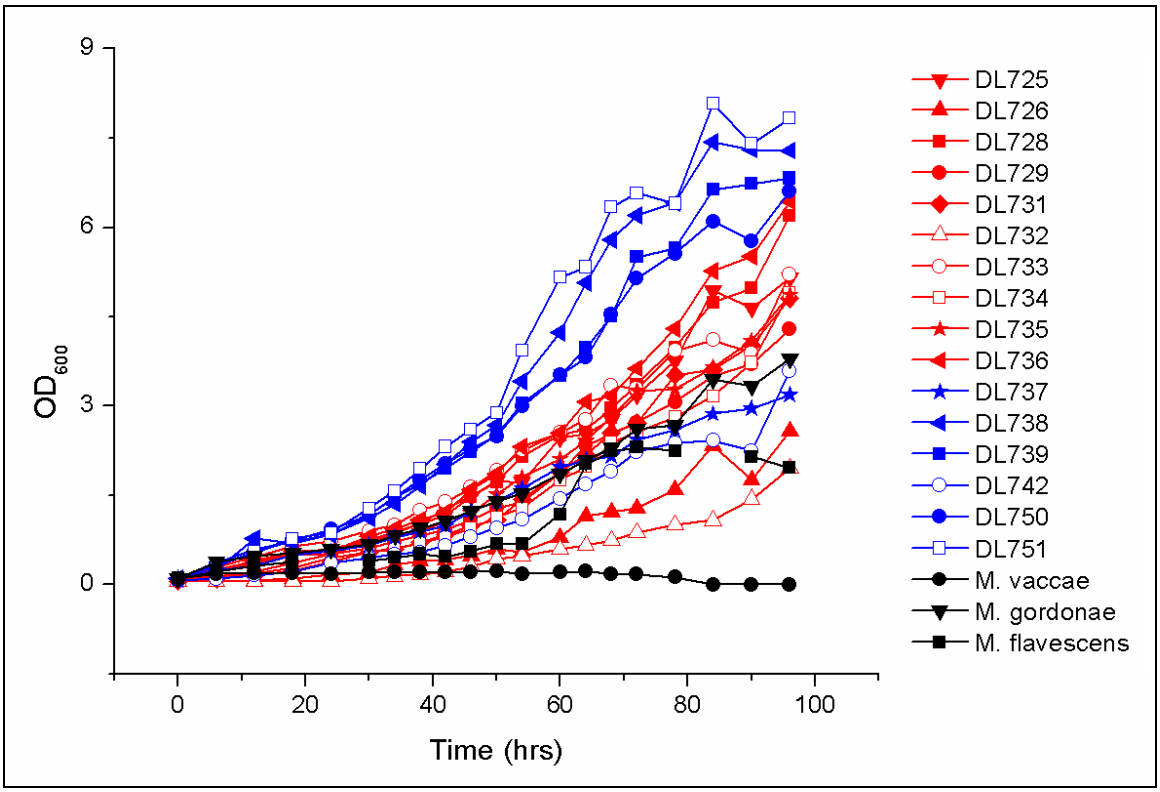

Figure A2. Growth curves of pitcher plant isolates, *M. gordonae, M. vaccae,* and *M. flavescens* at 28°C. Growth curves were conducted in 7H9 for five days and OD_{600} readings were taken every 4-6 hrs. Blue, LEC isolates; red, Repose Lake isolates; black, reference strains.

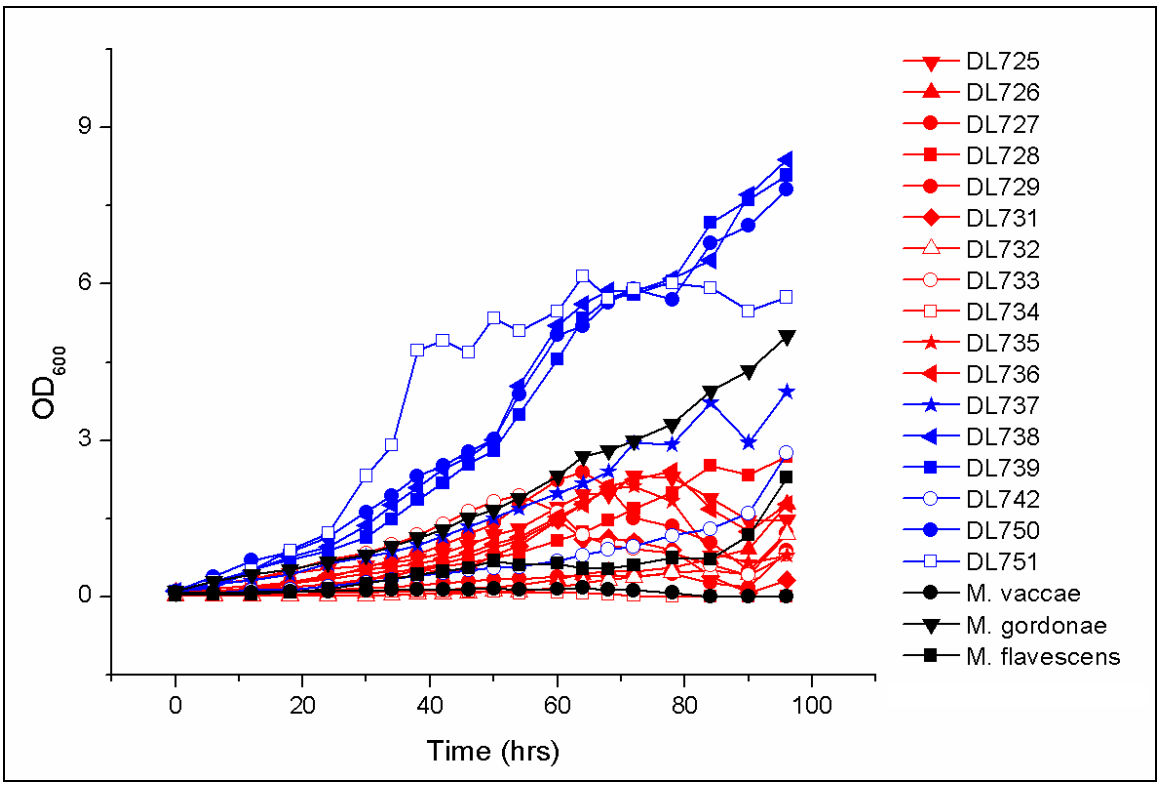

Figure A3. Growth curves of pitcher plant isolates, *M. gordonae, M. vaccae,* and *M. flavescens* at 32°C. Growth curves were conducted in 7H9 for five days and OD_{600} readings were taken every 4-6 hrs. Blue, LEC isolates; red, Repose Lake isolates; black, reference strains.

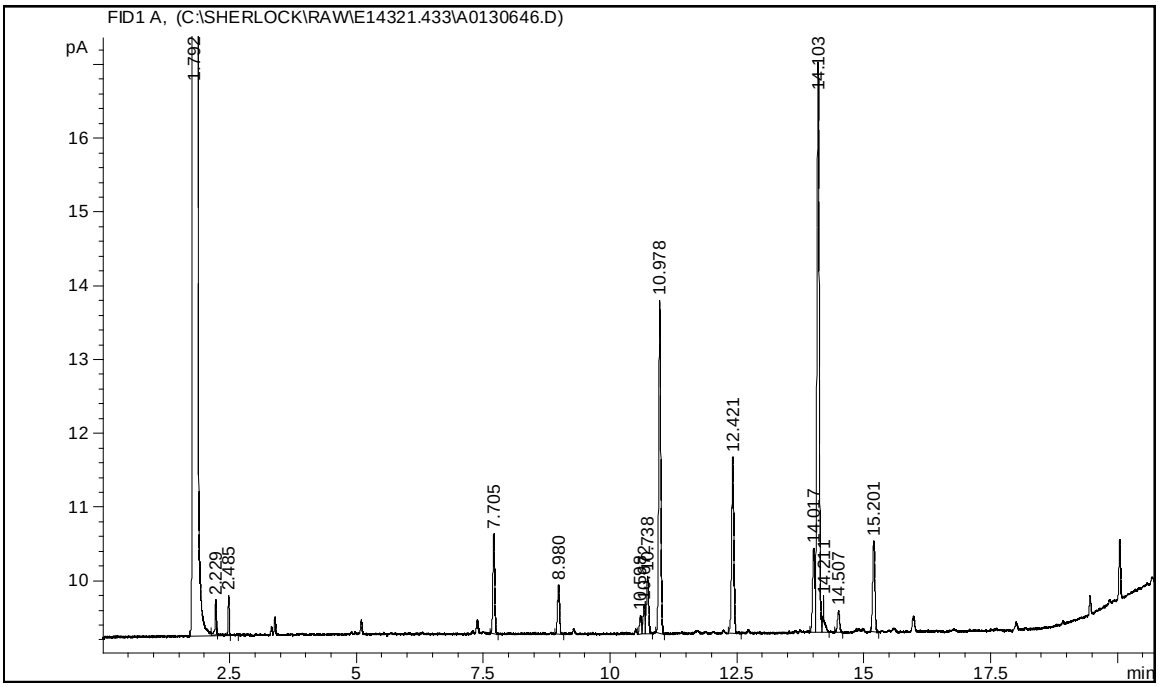

Figure A4. DL729 FAME chromatograph. FAME analysis was conducted at Microbial ID, Inc. (Newark, DE) by saponification, methylation, and extraction. Profiles were separated by gas chromatography, and identified using the Sherlock Microbial Identification System (MIDI, Inc.).

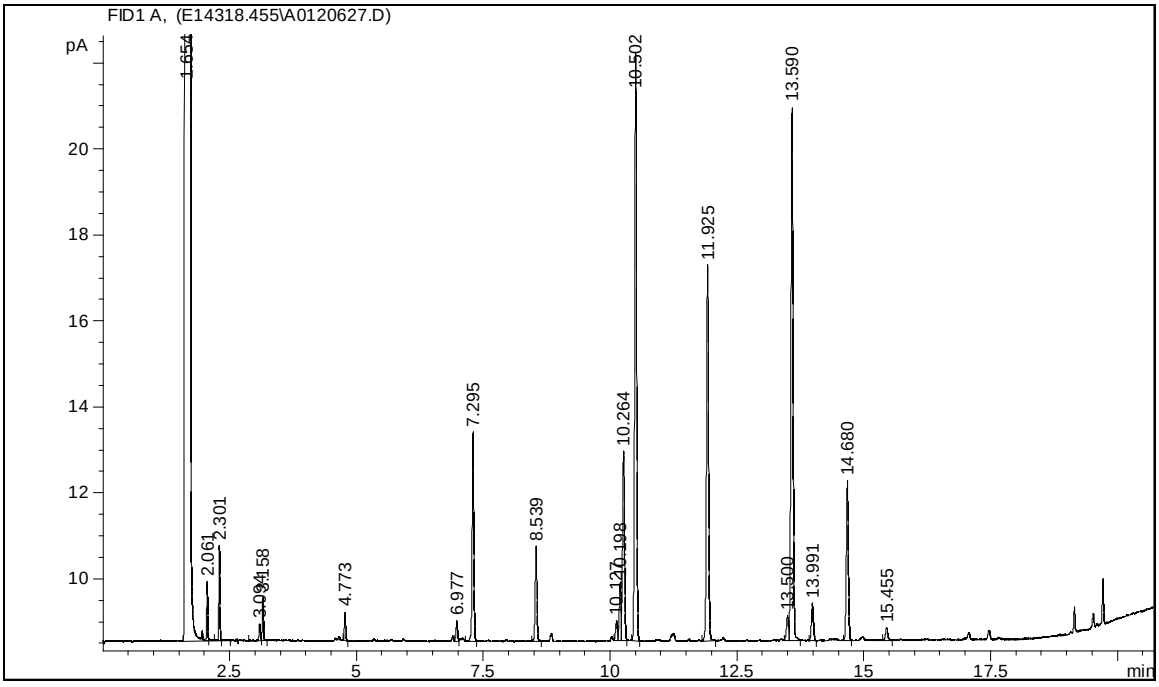

Figure A5. FAME chromatograph for DL730. FAME analysis was conducted at Microbial ID, Inc. (Newark, DE) by saponification, methylation, and extraction. Profiles were separated by gas chromatography, and identified using the Sherlock Microbial Identification System (MIDI, Inc.).

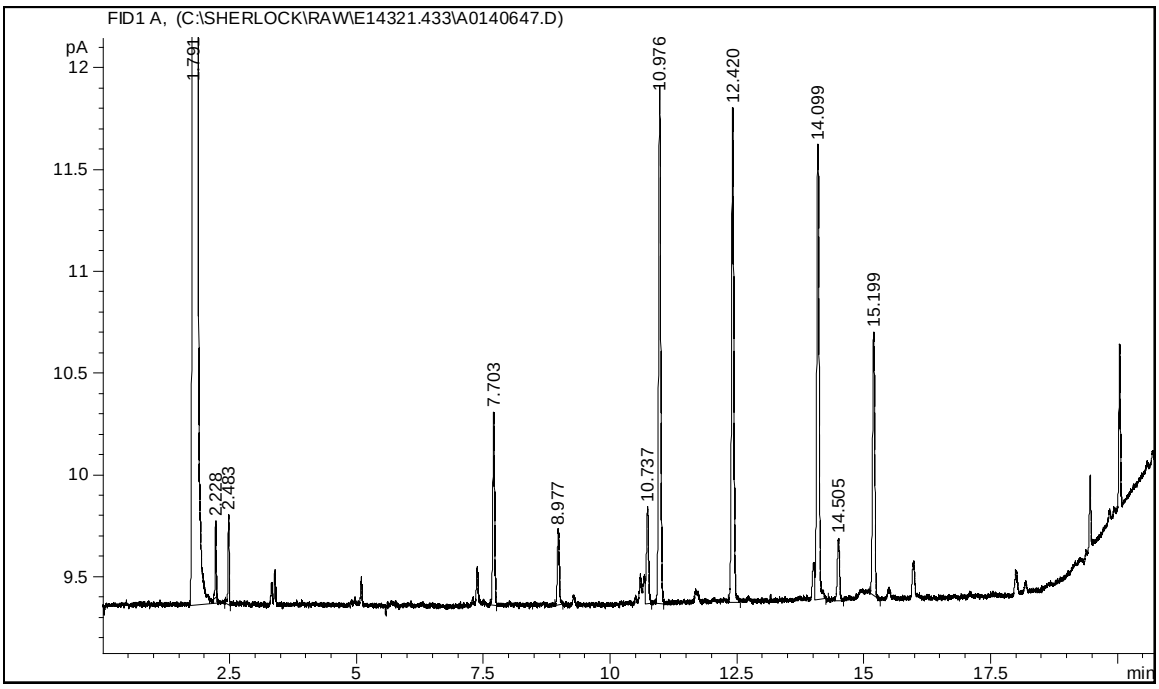

Figure A6. FAME chromatograph for DL731. FAME analysis was conducted at Microbial ID, Inc. (Newark, DE) by saponification, methylation, and extraction. Profiles were separated by gas chromatography, and identified using the Sherlock Microbial Identification System (MIDI, Inc.).

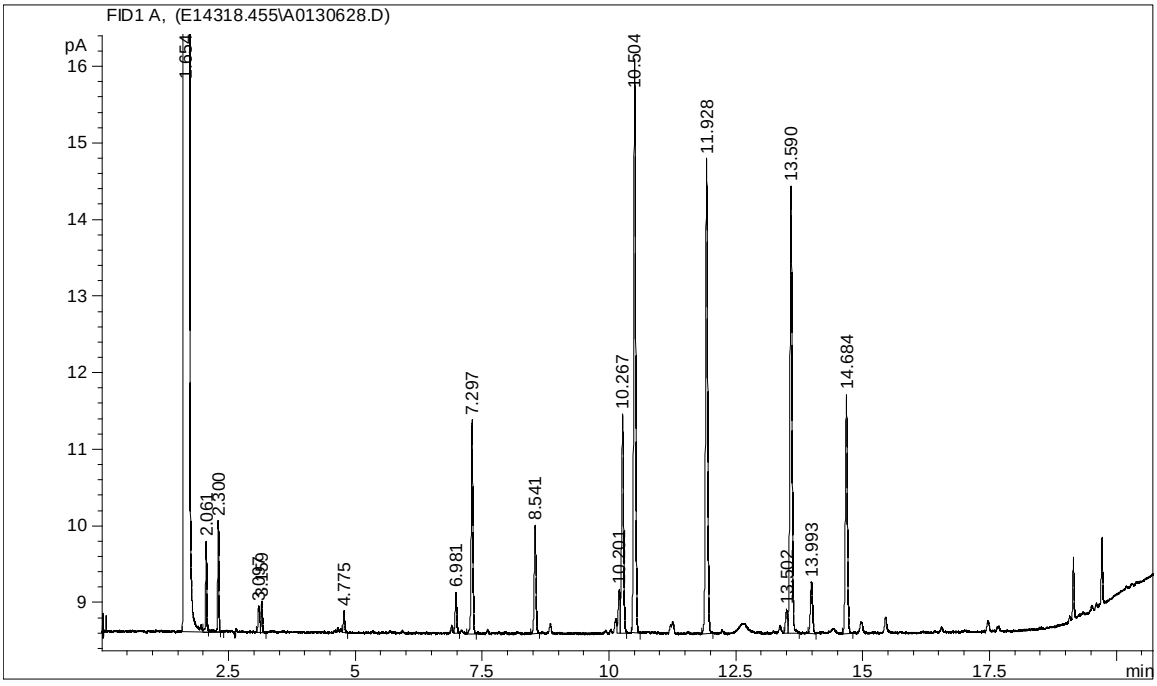

Figure A7. FAME chromatograph for DL734. FAME analysis was conducted at Microbial ID, Inc. (Newark, DE) by saponification, methylation, and extraction. Profiles were separated by gas chromatography, and identified using the Sherlock Microbial Identification System (MIDI, Inc.).

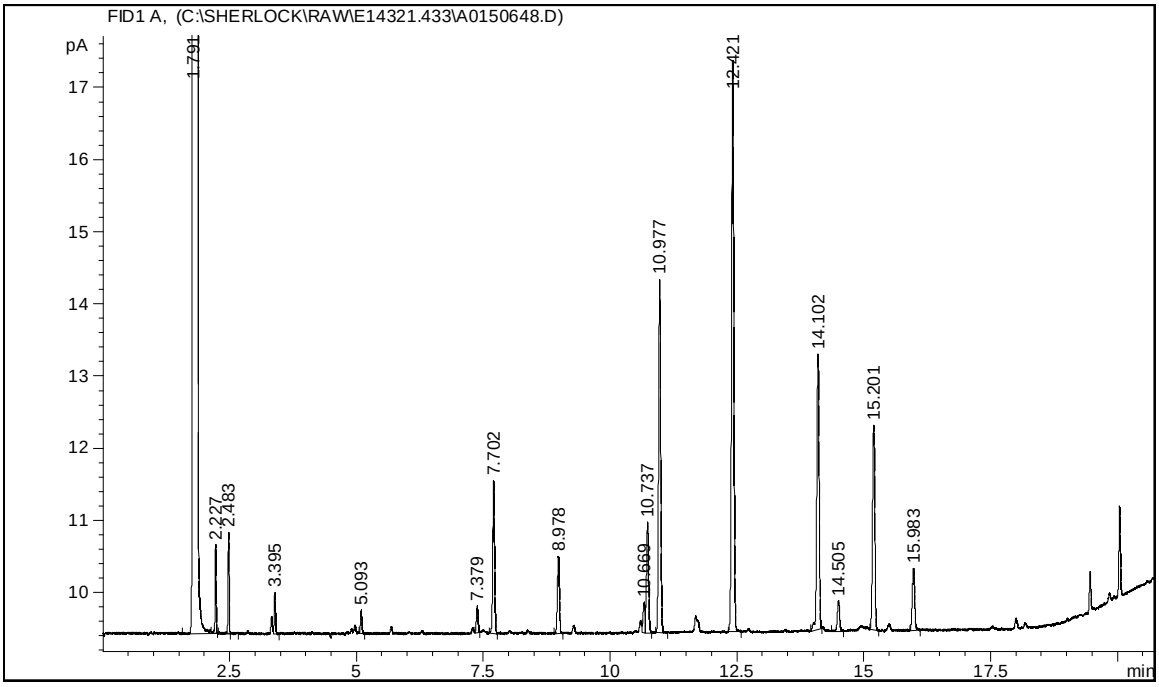

Figure A8. FAME chromatograph for DL736. FAME analysis was conducted at Microbial ID, Inc. (Newark, DE) by saponification, methylation, and extraction. Profiles were separated by gas chromatography, and identified using the Sherlock Microbial Identification System (MIDI, Inc.).

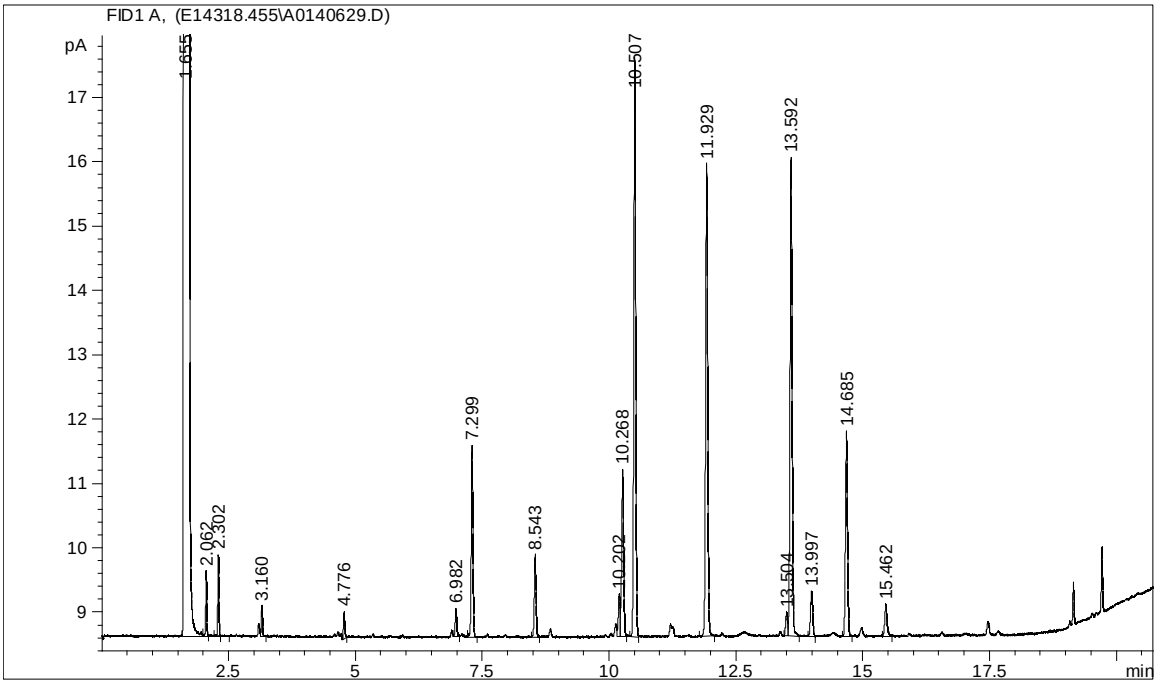

Figure A9. FAME chromatograph for DL737. FAME analysis was conducted at Microbial ID, Inc. (Newark, DE) by saponification, methylation, and extraction. Profiles were separated by gas chromatography, and identified using the Sherlock Microbial Identification System (MIDI, Inc.).

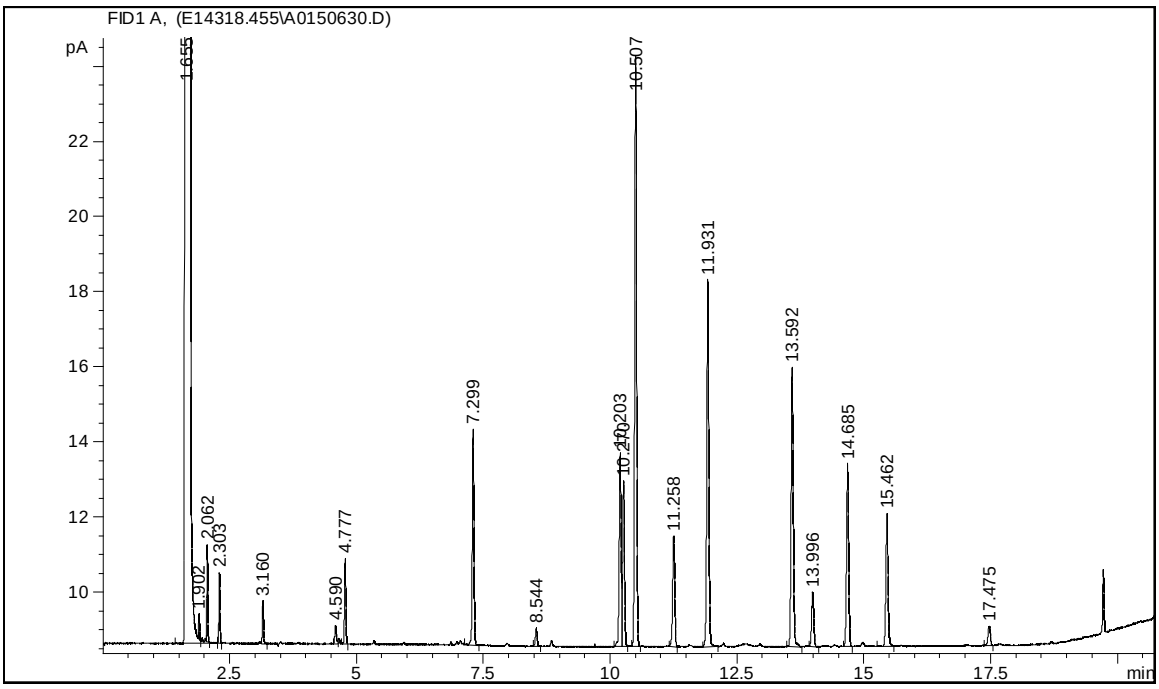

Figure A10. FAME chromatograph for DL738. FAME analysis was conducted at Microbial ID, Inc. (Newark, DE) by saponification, methylation, and extraction. Profiles were separated by gas chromatography, and identified using the Sherlock Microbial Identification System (MIDI, Inc.).

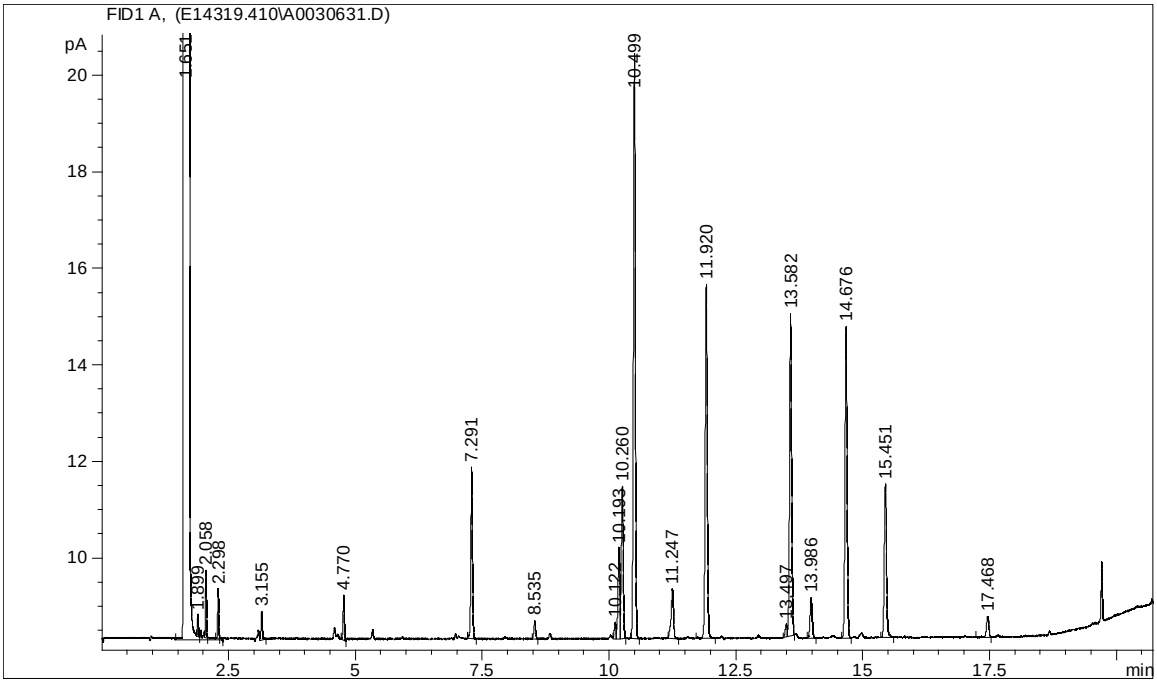

Figure A11. FAME chromatograph for DL739. FAME analysis was conducted at Microbial ID, Inc. (Newark, DE) by saponification, methylation, and extraction. Profiles were separated by gas chromatography, and identified using the Sherlock Microbial Identification System (MIDI, Inc.).

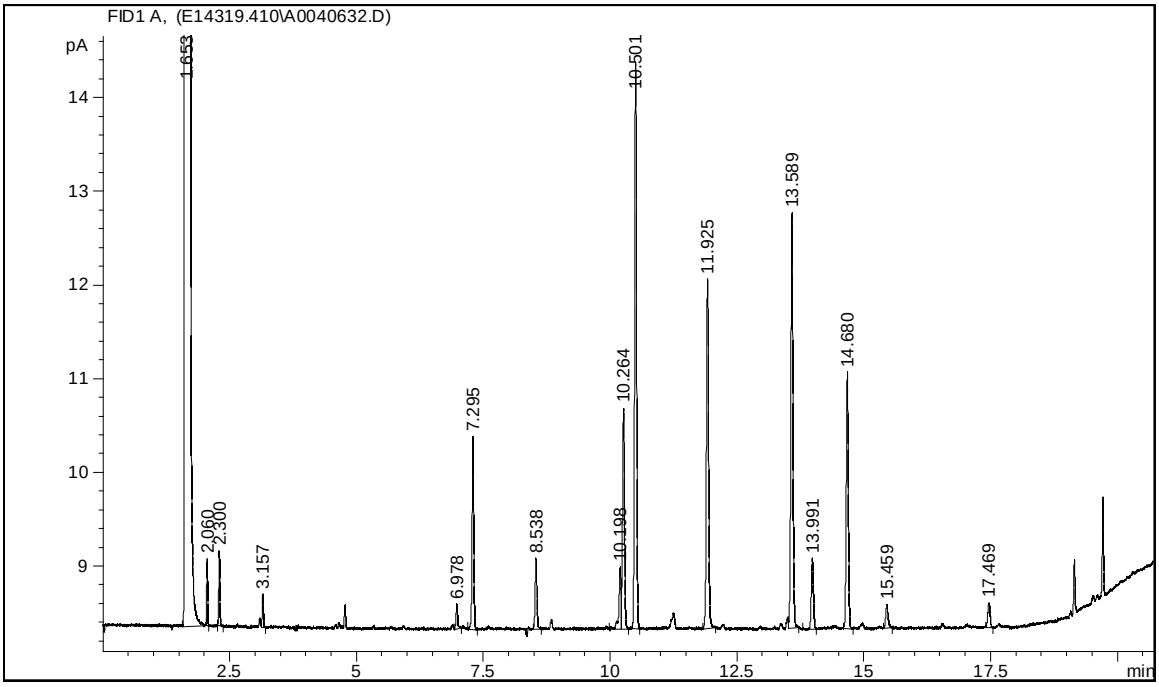

Figure A12. FAME chromatograph for DL742. FAME analysis was conducted at Microbial ID, Inc. (Newark, DE) by saponification, methylation, and extraction. Profiles were separated by gas chromatography, and identified using the Sherlock Microbial Identification System (MIDI, Inc.).

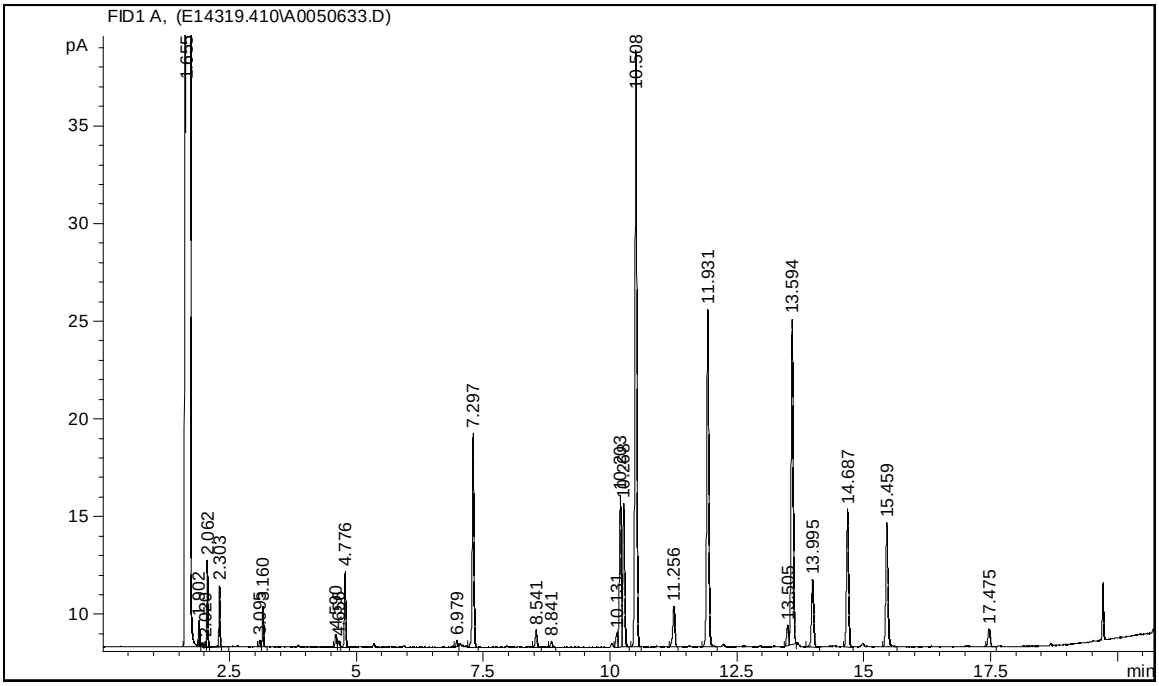

Figure A13. FAME chromatograph for DL750. FAME analysis was conducted at Microbial ID, Inc. (Newark, DE) by saponification, methylation, and extraction. Profiles were separated by gas chromatography, and identified using the Sherlock Microbial Identification System (MIDI, Inc.).

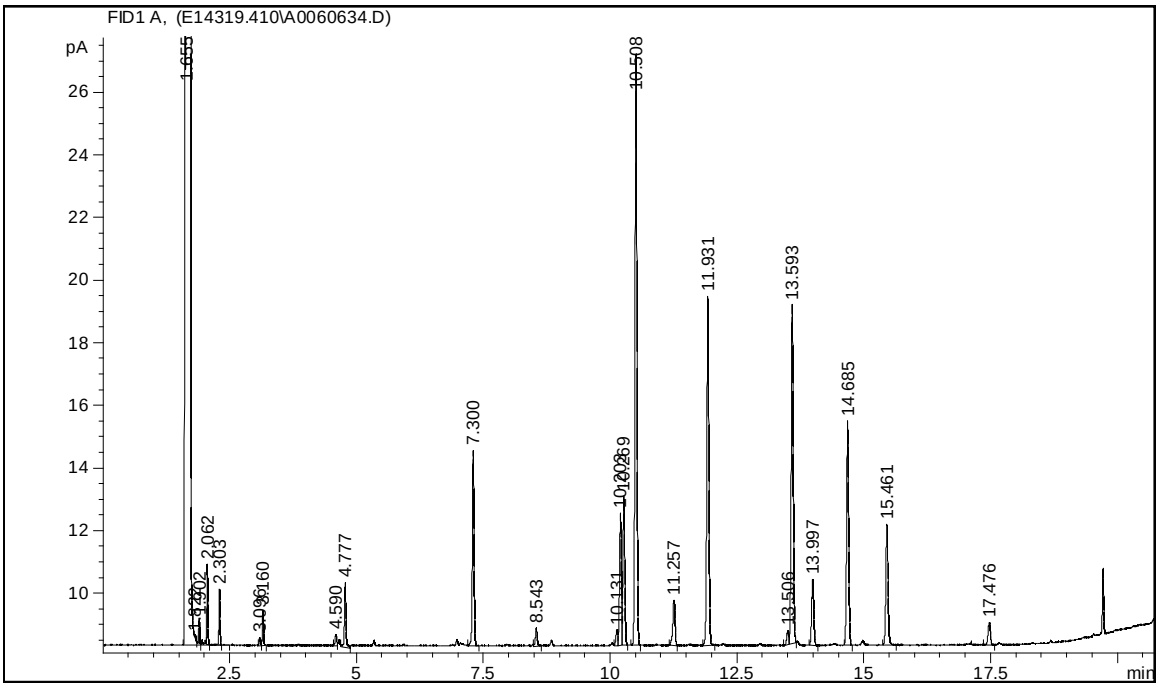

Figure A14. DL751 FAME chromatograph. FAME analysis was conducted at Microbial ID, Inc. (Newark, DE) by saponification, methylation, and extraction. Profiles were separated by gas chromatography, and identified using the Sherlock Microbial Identification System (MIDI, Inc.).

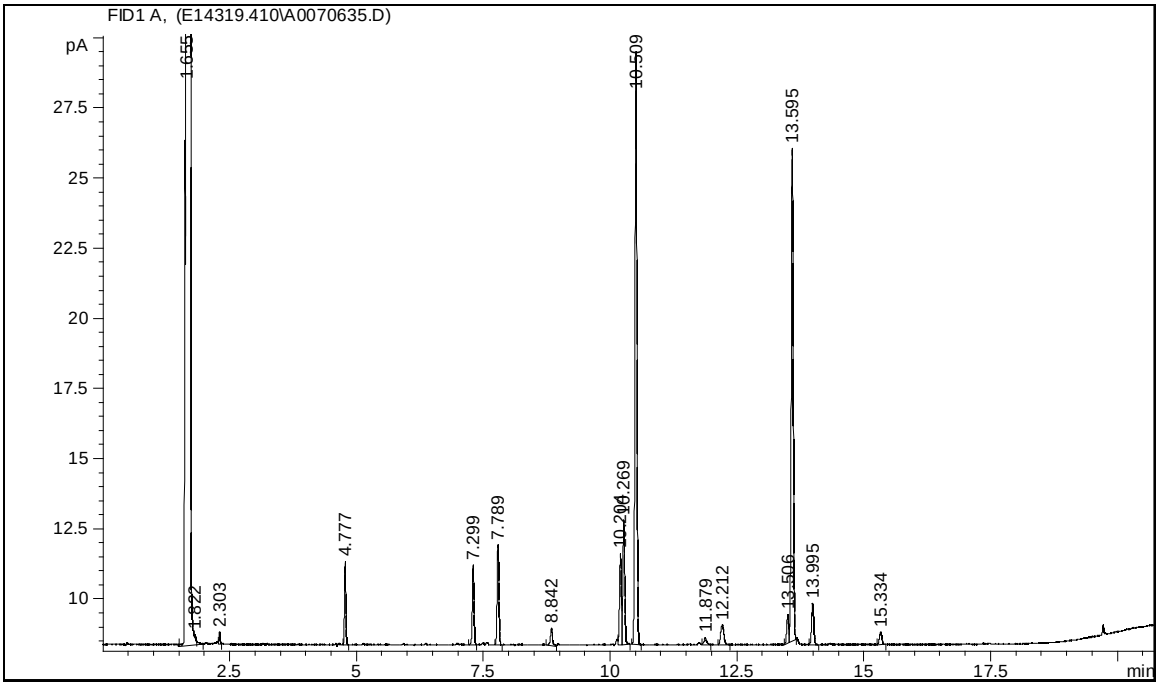

Figure A15. *M. gordonae* FAME chromatograph. FAME analysis was conducted at Microbial ID, Inc. (Newark, DE) by saponification, methylation, and extraction. Profiles were separated by gas chromatography, and identified using the Sherlock Microbial Identification System (MIDI, Inc.).

Appendix C. Supporting Figures for Chapter 4

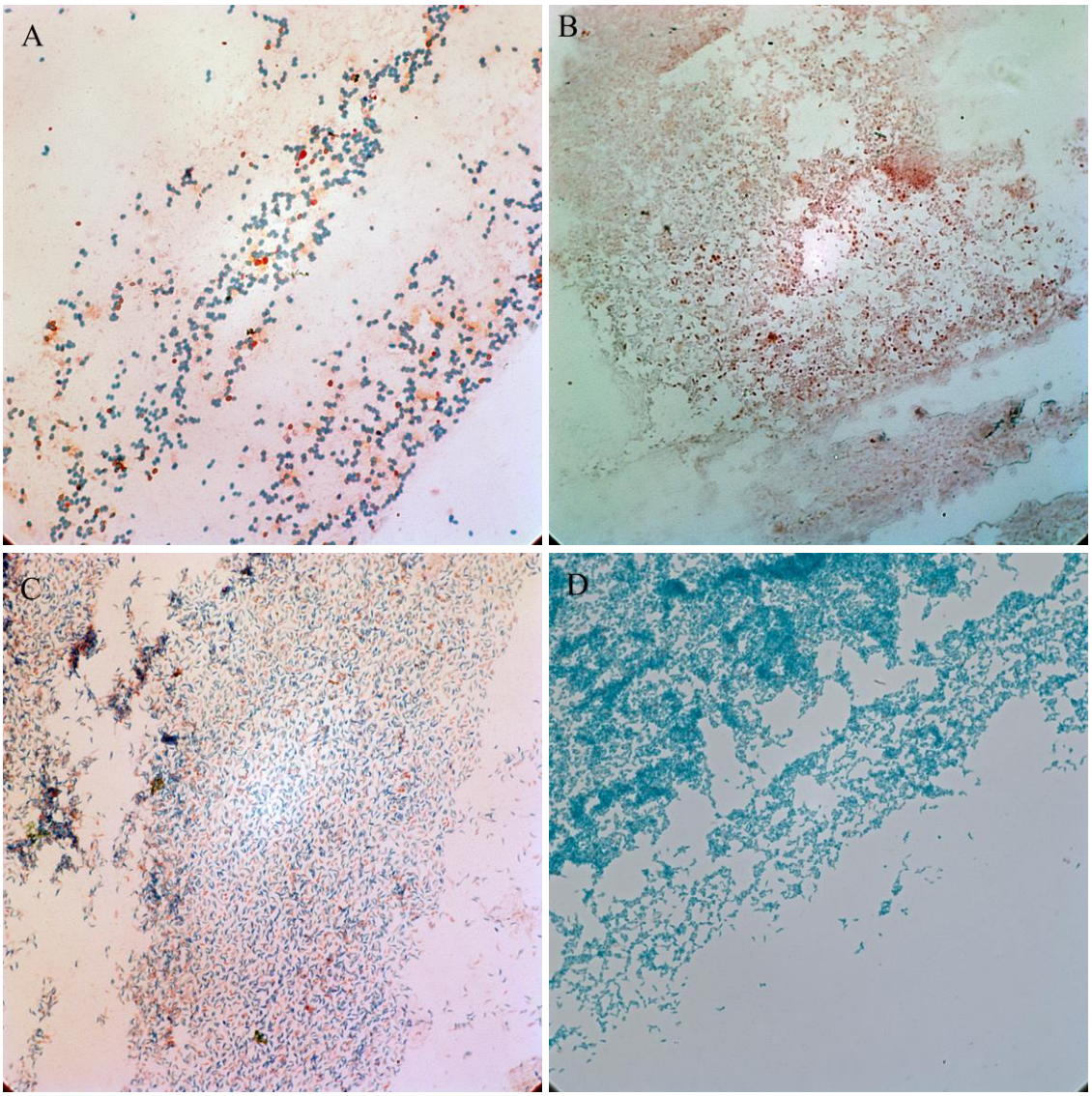

Figure A16. Endospore stain for *B. subtilis*, *E. coli*, and pitcher plant isolates. Malachite green endospore stain on one week old cells. **A,** *B. subtilis* exhibiting endospores, free spores and few vegetative cells. **B,** *E. coli* cells exhibiting no sporulation. **C,** DL725 stained green and red from malachite green and safranin. Cell morphology is unchanged. **D,** DL727 stained completely green. Cells do not exhibit spore-like structures similar to *B. subtilis*. **E,** DL750 did not stain malachite green positive. **F,** DL751 stained malachite green positive with homogenous cells.

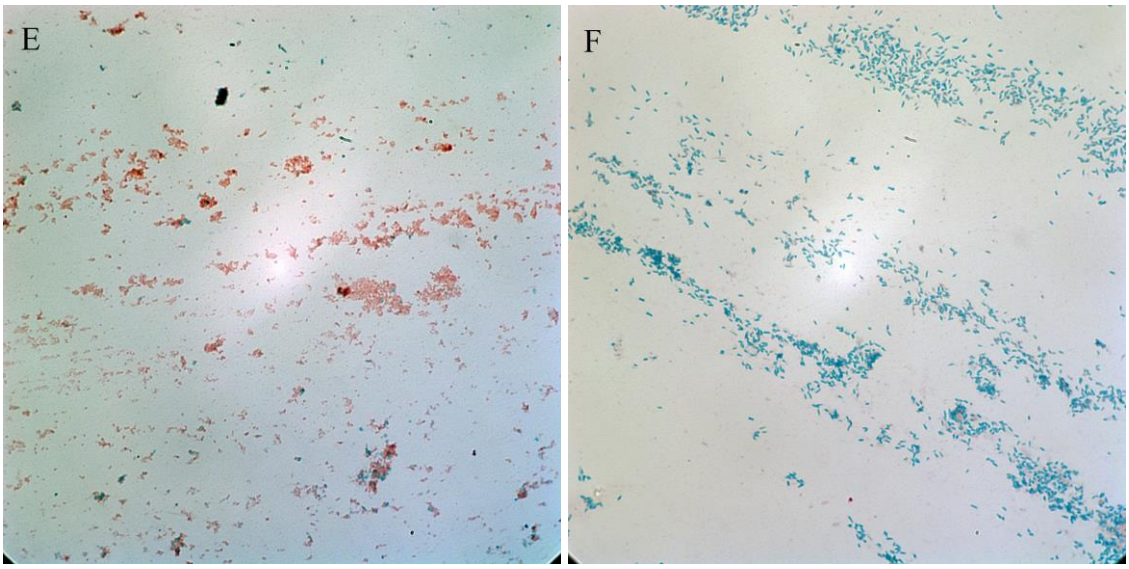

Figure A16 continued. Endospore stain for *B. subtilis, E. coli,* and pitcher plant isolates. Malachite green endospore stain on one week old cells. **A,** *B. subtilis* exhibiting endospores, free spores and few vegetative cells. **B,** *E. coli* cells exhibiting no sporulation. **C,** DL725 stained green and red from malachite green and safranin. Cell morphology is unchanged. **D,** DL727 stained completely green. Cells do not exhibit spore-like structures similar to *B. subtilis*. **E,** DL750 did not stain malachite green positive. **F,** DL751 stained malachite green positive with homogenous cells.